(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 March 2004 (11.03.2004)

PCT

(10) International Publication Number
WO 2004/020637 A1

(51) International Patent Classification⁷: C12N 15/53

(21) International Application Number: PCT/AU2003/001111

(22) International Filing Date: 29 August 2003 (29.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

2002951088	30 August 2002 (30.08.2002)	AU
2002952835	16 September 2002 (16.09.2002)	AU

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/020637 A1

(54) Title: FLAVANOID 3',5'HYDROXYLASE GENE SEQUENCES AND USES THEREFOR

(57) Abstract: The present invention relates generally to a genetic sequence encoding a polypeptide having flavonoid 3', 5'-hydroxylase (F3'5'H) activity and to the use of the genetic sequence and/or its corresponding polypeptide thereof *inter alia* to manipulate color in flowers or parts thereof or in other plant tissue. More particularly, the F3'5'H has the ability to modulate dihydrokaempferol (DHK) metabolism as well as the metabolism of other substrates such as dihydroquercetin (DHQ), naringenin and eriodictyol. Even more particularly, the present invention provides a genetic sequence encoding a polypeptide having F3'5'H activity when expressed in rose or gerbera or botanically related plants. The instant invention further relates to antisense and sense molecules or RNAi-inducing molecules corresponding to all or part of the subject genetic sequence or a transcript thereof. The present invention further relates to promoters which operate efficiently in plants such as rose, gerbera or botanically related plants.

FLAVONOID 3',5' HYDROXYLASE GENE SEQUENCES AND USES TEREFOR

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a genetic sequence encoding a polypeptide having flavonoid 3', 5'-hydroxylase (F3'5'H) activity and to the use of the genetic sequence and/or its corresponding polypeptide thereof *inter alia* to manipulate color in flowers or parts thereof or in other plant tissue. More particularly, the F3'5'H has the ability to modulate dihydrokaempferol (DHK) metabolism as well as the metabolism of other substrates such as dihydroquercetin (DHQ), naringenin and eriodictyol. Even more particularly, the present invention provides a genetic sequence encoding a polypeptide having F3'5'H activity when expressed in rose or gerbera or botanically related plants. The instant invention further relates to antisense and sense molecules or RNAi-inducing molecules corresponding to all or part of the subject genetic sequence or a transcript thereof as well as to genetically modified plants as well as cut flowers, parts and reproductive tissue from such plants. The present invention further relates to promoters which operate efficiently in plants such as rose, gerbera or botanically related plants.

DESCRIPTION OF PRIOR ART

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

The flower or ornamental plant industry strives to develop new and different varieties of flowers and/or plants. An effective way to create such novel varieties is through the manipulation of flower color. Classical breeding techniques have been used with some success to produce a wide range of colors for almost all of the commercial varieties of flowers and/or plants available today. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for a single species to have the full spectrum of colored varieties. For example, the development of novel colored varieties of plants or plant parts such as flowers, foliage and stems would offer a significant opportunity in both the cut flower and ornamental markets. In the flower or ornamental plant industry, the development of novel colored varieties of major flowering species such as rose, chrysanthemum, tulip, lily, carnation, gerbera, orchid, lisianthus, begonia, torenia, geranium, petunia, nierembergia, pelargonium, iris, impatiens and cyclamen would be of great interest. A more specific example would be the development of a blue rose or gerbera for the cut flower market.

In addition, the development of novel colored varieties of plant parts such as vegetables, fruits and seeds would offer significant opportunities in agriculture. For example, novel colored seeds would be useful as proprietary tags for plants. Furthermore modifications to flavonoids common to berries or fruits including grapes and apples and their juices including wine have the potential to impart altered style characteristics of value to such fruit and byproduct industries.

Flower color is predominantly due to three types of pigment: flavonoids, carotenoids and betalains. Of the three, the flavonoids are the most common and contribute a range of colors from yellow to red to blue. The flavonoid molecules that make the major contribution to flower color are the anthocyanins, which are glycosylated derivatives of cyanidin and its methylated derivative peonidin, *delphinidin or delphinidin-based molecules* and its methylated derivatives petunidin and malvidin and pelargonidin. Anthocyanins are localised in the vacuole of the epidermal cells of petals or the vacuole of the sub epidermal cells of leaves.

The flavonoid pigments are secondary metabolites of the phenylpropanoid pathway. The biosynthetic pathway for the flavonoid pigments (flavonoid pathway) is well established, (Holton and Cornish, *Plant Cell 7*: 1071-1083, 1995; Mol *et al.*, *Trends Plant Sci. 3*: 212-217, 1998; Winkel-Shirley, *Plant Physiol. 126*: 485-493, 2001a; and Winkel-Shirley, *Plant Physiol. 127*: 1399-1404, 2001b) and is shown in Figures 1A and B. Three reactions and enzymes are involved in the conversion of phenylalanine to *p*-coumaroyl-CoA, one of the first key substrates in the flavonoid pathway. The enzymes are phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA (provided by the action of acetyl CoA carboxylase (ACC) on acetyl CoA and CO_2) with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6', tetrahydroxy-chalcone, is normally rapidly isomerized by the enzyme chalcone flavanone isomerase (CHI) to produce naringenin. Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The pattern of hydroxylation of the B-ring of dihydrokaempferol (DHK) plays a key role in determining petal color. The B-ring can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) or dihydromyricetin (DHM), respectively. Two key enzymes involved in this part of the pathway are flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase, both of the cytochrome P450 class of enzymes. Cytochrome P450 enzymes are widespread in nature and genes have been isolated and sequenced from vertebrates, insects, yeasts, fungi, bacteria and plants.

Flavonoid 3'-hydroxylase (F3'H) is a key enzyme in the flavonoid pathway leading to the cyanidin- based pigments which, in many plant species (for example *Rosa spp.*, *Dianthus spp.*, *Petunia spp.*, begonia, cyclamen, impatiens, morning glory and chrysanthemum), contribute to red and pink flower color.

Flavonoid 3', 5'-hydroxylase (F3'5'H) is a key enzyme in the flavonoid pathway leading to the delphinidin- based pigments which, in many plant species (for example, *Petunia spp.*,

Viola spp., Lisianthus spp., Gentiana spp., Sollya spp., Salvia spp., Clitoria spp., Kennedia spp., Campanula spp., Lavandula spp., Verbena spp., Torenia spp., Delphinium spp., Solanum spp., Cineraria spp., Vitis spp., Babiana stricta, Pinus spp., Picea spp., Larix spp., Phaseolus spp., Vaccinium spp., Cyclamen spp., Iris spp., Pelargonium sp., Liparieae, *Geranium spp., Pisum spp., Lathyrus spp., Catharanthus spp., Malvia spp., Mucuna spp., Vicia spp., Saintpaulia spp., Lagerstroemia spp., bouchina spp., Plumbago spp., Hypocalyptus spp., Rhododendron spp., Linum spp., Macroptilium spp., Hibiscus spp., Hydrangea spp., Cymbidium spp., Millettia spp., Hedysarum spp., Lespedeza spp., Asparagus spp. Antigonon spp., Pisum spp., Freesia spp., Brunella spp., Clarkia spp.*, etc.), contribute to purple and blue flower color. Many plant species such as roses, gerberas, chrysanthemums and carnations, do not produce delphinidin-based pigments because they lack a F3'5'H activity.

The next step in the pathway, leading to the production of the colored anthocyanins from the dihydroflavonols (DHK, DHQ, DHM), involves dihydroflavonol-4-reductase (DFR) leading to the production of the leucoanthocyanidins. The leucoanthocyanidins are subsequently converted to the anthocyanidins, pelargonidin, cyanidin and *delphinidin or delphinidin-based molecules*. These flavonoid molecules are unstable under normal physiological conditions and glycosylation at the 3-position, through the action of glycosyltransferases, stabilizes the anthocyanidin molecule thus allowing accumulation of the anthocyanins. In general, the glycosyltransferases transfer the sugar moieties from UDP sugars to the flavonoid molecules and show high specificities for the position of glycosylation and relatively low specificities for the acceptor substrates (Seitz and Hinderer, Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants*. Constabel, F. and Vasil, I.K. (eds.), Academic Press, New York, USA, *5:* 49-76, 1988). Anthocyanins can occur as 3-monosides, 3-biosides and 3-triosides as well as 3, 5-diglycosides and 3, 7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose (Strack and Wray, In: *The Flavonoids - Advances in Research since 1986*. Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

Glycosyltransferases involved in the stabilisation of the anthocyanidin molecule include UDP glucose: flavonoid 3-glucosyltransferase (3GT), which transfers a glucose moiety from UDP glucose to the 3-*O*-position of the anthocyanidin molecule to produce anthocyanidin 3-*O*-glucoside.

In petunia and pansy (amongst others), anthocyanidin 3-*O*-glucoside are generally glycosylated by another glycosyltransferase, UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase (3RT), which adds a rhamnose group to the 3-*O*-bound glucose of the anthocyanin molecule to produce the anthocyanidin 3-rutinosides, and once acylated, can be further modified by UDP: glucose anthocyanin 5 glucosyltransferase (5GT). However, in roses (amongst others), the anthocyanidin 3-*O*-glucosides are generally glycosylated by another glycosyltransferase, UDP: glucose anthocyanin 5 glucosyltransferase (5GT) to produce anthocyanidin 3, 5 diglucosides.

Many anthocyanidin glycosides exist in the form of acylated derivatives. The acyl groups that modify the anthocyanidin glycosides can be divided into two major classes based upon their structure. The aliphatic acyl groups include malonic acid or succinic acid and the aromatic class include the hydroxy cinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid and the benzoic acids such as *p*-hydroxybenzoic acid.

Methylation at the 3' and 5' positions of the B-ring of anthocyanidin glycosides can also occur. Methylation of cyanidin-based pigments leads to the production of peonidin. Methylation of the 3' position of delphinidin-based pigments results in the production of petunidin, whilst methylation of the 3' and 5' positions results in malvidin production. Methylation of malvidin can also occur at the 5-O and 7-O positions to produce capensinin (5-O-methyl malvidin) and 5, 7-di-O-methyl malvidin.

In addition to the above modifications, pH of the vacuole or compartment where pigments are localised and copigmentation with other flavonoids such as flavonols and flavones can affect petal color. Flavonols and flavones can also be aromatically acylated (Brouillard and

Dangles, In: *The Flavonoids -Advances in Research since 1986*. Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

The ability to control F3'5'H activity, or other enzymes involved in the flavonoid pathway, in flowering plants would provide a means of manipulating the color of plant parts such as petals, fruit, leaves, sepals, seeds etc. Different colored versions of a single cultivar could thereby be generated and in some instances a single species would be able to produce a broader spectrum of colors.

Two nucleotide sequences (referred to herein as SEQ ID NO:1 and SEQ ID NO:3) encoding petunia F3'5'Hs have been cloned (see International Patent Application No. PCT/AU92/00334 and Holton *et al.*, *Nature*, *366*: 276-279, 1993a). These sequences were efficient in modulating 3', 5' hydroxylation of flavonoids in petunia (see International Patent Application No. PCT/AU92/00334 incorporated herein by reference and Holton *et al.*, 1993a, *supra*), tobacco (see International Patent Application No. PCT/AU92/00334 incorporated herein by reference) and carnations (see International Patent Application No. PCT/AU96/00296 incorporated herein by reference). Surprisingly, however, inclusion of these sequences in standard expression cassettes, did not lead to the production of intact or full-length transcripts as detectable by RNA or Northern blot analysis and consequently 3', 5'-hydroxylated flavonoids were not produced in roses. There is a need, therefore, to identify further genetic sequences encoding F3'5'Hs which efficiently accumulate and are then able to modulate 3', 5' hydroxylation of flavonoids such as anthocyanins in roses and other key commercial plant species.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc.

Genetic sequences encoding a F3'5'H have been identified and cloned from a number of plant species. The F3'5'H genetic sequences when expressed in rose petal tissue results in detectable level of delphinidin or delphinidin-based molecules as determined by a chromatographic technique such as thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Alternatively, or in addition, expression of the genetic sequences in rose petal tissue results in a sufficient level and length of transcript which is capable of being translated to F3'5'H. This is conveniently measured as delphinidin or delphinidin-based molecules, detectable using a chromatographic technique such as TLC or HPLC. The genetic sequences of the present invention permit the modulation of expression of genes encoding this enzyme by, for example, *de novo* expression, over-expression, suppression, antisense inhibition, ribozyme activity, RNAi-induction or methylation-induction. The ability to control F3'5'H synthesis in plants and more specifically in roses or gerberas permits modulation of the composition of individual anthocyanins as well as alteration of relative levels of flavonols and anthocyanins, thereby enabling the manipulation of color of tissues and/or organs of plants such as petals, leaves, seeds, sepals, fruits etc.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a flavonoid 3', 5' hydroxylase (F3'5'H) or a polypeptide having F3'5'H activity

wherein expression of said nucleic acid molecule in a rose petal tissue results in detectable levels of delphinidin or delphinidin-based molecules as measured by a chromatographic technique.

Another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in a sufficient level and length of transcript which is translated to said F3'5'H as determined by detectable levels of delphinidin or delphinidin-based molecules as measured by a chromatographic technique.

The isolated nucleic acid molecule of the present invention, therefore, encodes a F3'5'H which is capable of more efficient conversion of DHK to DHM in roses than is the F3'5'H encoded by the nucleotide sequence set forth in SEQ ID NO:1 and SEQ ID NO:3 as measured by delphinidin production in rose petals.

Efficiency as used herein relates to the capability of the F3'5'H enzyme to convert its substrate DHK or DHQ into DHM in a rose cell (or any cell of a commercially important plant such as gerbera). This conversion provides the plant with a substrate (DHM) for other enzymes of the flavonoid pathway which are present in the palnt to further modify the substrate. This modification may include for example, glycosylation, acylation, rhamnosylation and/or methylation, to produce various anthocyanins which contribute to the production of a range of colors. The modulation of 3',5'-hydroxylated anthocyanins in rose is thereby enabled. Efficiency is conveniently assessed by one or more parameters selected from: extent of F3'5'H transcription, as determined by the amount of intact F3'5'H mRNA produced (as detected by Northern blot analysis); extent of translation of the F3'5'H mRNA, as determined by the amount of translation product produced; extent of F3'5'H enzyme activity as determined by the production of anthocyanin derivatives of DHQ or DHM including delphinidin or delphinidin-based pigments (as detected by TLC or HPLC); the extent of effect on flower color.

It has also been surprisingly determined that certain combinations of promoter and *F3'5'H* gene sequences that were functional in carnation and petunia were not functional in rose. Surprisingly, only a particular subset of promoter and *F3'5'H* gene sequence combinations resulted in 3', 5'-hydroxylated flavonoids in rose flowers. These included *F3'5'H* sequences isolated from *Viola spp.*, *Salvia spp. Lavandula spp.* and *Sollya spp.* Furthermore, the *Viola F3'5'H* (or pansy *F3'5'H*) sequences were found to result in the highest accumulation of 3', 5'-hydroxylated flavonoids in rose. The novel promoter and *F3'5'H* gene sequence combinations can be employed *inter alia* to modulate the color or flavour or other characteristics of plants or plant parts such as but not limited to flowers, fruits, nuts, roots, stems, leaves or seeds. Thus, the present invention represents a new approach to developing plant varieties having altered color characteristics. Other uses include, for example, the production of novel extracts of F3'5'H transformed plants wherein the extract has use, for example, as a flavouring or food additive or health product or beverage or juice or coloring. Beverages may include but are not limited to wines, spirits, teas, coffee, milk and dairy products.

In a preferred embodiment, therefore, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding pansy F3'5'H, salvia F3'5'H, lavender F3'5'H, kennedia F3'5'H or sollya F3'5'H or a functional derivative of the enzyme.

The nucleotide sequences encoding the pansy F3'5'H (SEQ ID NOs:9 and 11), salvia F3'5'H (SEQ ID NOs:13 and 15), sollya F3'5'H (SEQ ID NO:17), lavender F3'5'H (SEQ ID NO:31) and kennedia F3'5'H (SEQ ID NO:26) are defined by sequence identifiers indicated in parentheses. A summary of the sequence identifiers is shown in Table 1.

Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or SEQ ID NO:31 (lavender) or SEQ ID NO:26 (kennedia) or having at least about 50% similarity thereto or capable of

hybridizing to the sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 under low stringency conditions.

The amino acid sequences of the preferred F3'5'H enzymes are set forth in SEQ ID NO:10 (pansy) or SEQ ID NO:12 (pansy) or SEQ ID NO:14 (salvia) or SEQ ID NO:16 (salvia) or SEQ ID NO:18 (sollya) or SEQ ID NO:32 (lavender) or SEQ ID NO:27 (kennedia).

A further aspect of the present invention provides a method for producing a transgenic flowering plant capable of synthesizing a F3'5'H said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said F3'5'H under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The expression of the nucleic acid sequence generally results in a transcription of sufficient level and length to encode a F3'5'H. This is conveniently determined by detectable levels of delphinidin or delphinidin-based molecules as measured by chromatographic techniques such as TLC or HPLC. The transgenic plant may thereby produce a non-indigenous F3'5'H at elevated levels relative to the amount expressed in a comparable non-transgenic plant. This generally results in a visually detectable color change in the plant or plant part or preferably in the inflorescence or flowers of said plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced F3'5'H activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced F3'5'H activity, said method comprising altering the F3'5'H gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Still another aspect of the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered floral or inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

Still a further aspect of the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered floral or inflorescence properties, said method comprising alteration of the *F3'5'H* gene through modification of nucleotide sequences *via* homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Even yet another aspect of the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding a F3'5'H or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule encoding said F3'5'H, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, a F3'5'H, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered floral or inflorescence properties.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered aerial parts of the plant such as fruit, berries, sepal, bract, petiole, peduncle, ovaries, anthers or stem properties.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in particular, the extracts from those transgenic plants when used as a flavouring or food additive or health product or beverage or juice or coloring.

A further aspect of the present invention is directed to recombinant forms of F3'5'H.

Another aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing a F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

Yet another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding a F3'5'H extrachromasomially in plasmid form.

Still another aspect of the present invention extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or SEQ ID NO:32 or SEQ ID NO:27 or an amino acid sequence having at least about 50% similarity to SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or SEQ ID NO:32 or SEQ ID NO:27 or a derivative of said polypeptide.

The present invention further provides promoters which operate efficiently in plants such as rose and gerbera or botanically related plants. Such promoters include a rose CHS promoter, chrysanthemum CHS promoter and a CaMV 35S promoter.

A summary of sequence identifiers used throughout the subject specification is provided in Table 1:

TABLE 1

Summary of sequence identifiers

SEQ ID NO:	NAME	SPECIES	TYPE OF SEQ	DESCRIPTION
1	*petHf1*.nt	*Petunia hybrida*	nucleotide	*F3'5'H cDNA*
2	*petHf1*.aa	*Petunia hybrida*	amino acid	*translation of F3'5'H cDNA*
3	*petHf2*.nt	*Petunia hybrida*	nucleotide	*F3'5'H cDNA*
4	*petHf2*.aa	*Petunia hybrida*	amino acid	*translation of F3'5'H cDNA*
5	*RoseCHS* promoter	*Rosa hybrida*	nucleotide	*promoter fragment*
6	D8 oligo#2	*Petunia hybrida*	nucleotide	*oligonucleotide*
7	D8 oligo #4	*Petunia hybrida*	nucleotide	*oligonucleotide*
8	chrysanCHSATG	chrysanthemum	nucleotide	*oligonucleotide*
9	*BP#18*.nt	*Viola spp.*	nucleotide	*F3'5'H cDNA*
10	*BP#18*.aa	*Viola spp.*	amino acid	*translation of F3'5'H cDNA*
11	*BP#40*.nt	*Viola spp.*	nucleotide	*F3'5'H cDNA*
12	*BP#40*.aa	*Viola spp.*	amino acid	*translation of F3'5'H cDNA*
13	*Sal#2*.nt	*Salvia spp.*	nucleotide	*F3'5'H cDNA*

SEQ ID NO:	NAME	SPECIES	TYPE OF SEQ	DESCRIPTION
14	*Sal#2*.aa	*Salvia spp.*	amino acid	*translation of F3'5'H cDNA*
15	*Sal#47*.nt	*Salvia spp.*	nucleotide	*F3'5'H cDNA*
16	*Sal#47*.aa	*Salvia spp.*	amino acid	*translation of F3'5'H cDNA*
17	*Soll#5*.nt	*Sollya spp.*	nucleotide	*F3'5'H cDNA*
18	*Soll#5*.aa	*Sollya spp.*	amino acid	*translation of F3'5'H cDNA*
19	FLS-Nco	*Petunia hybrida*	nucleotide	*oligonucleotide*
20	*BpeaHF2*.nt	*Clitoria ternatea*	nucleotide	*F3'5'H cDNA*
21	*BpeaHF2*.aa	*Clitoria ternatea*	amino acid	*translation of F3'5'H cDNA*
22	*Gen#48*.nt	*Gentiana triflora*	nucleotide	*F3'5'H cDNA*
23	*Gen#48*.aa	*Gentiana triflora*	amino acid	*translation of F3'5'H cDNA*
24	*PetD8 5'*	*Petunia hybrida*	nucleotide	*oligonucleotide*
25	*Bpea* primer	*Clitoria ternatea*	nucleotide	*oligonucleotide*
26	*Kenn#31*.nt	*Kennedia spp.*	nucleotide	*F3'5'H cDNA*
27	*Kenn#31*.aa	*Kennedia spp.*	amino acid	*translation of F3'5'H cDNA*
28	*chrysCHS*.nt	chrysanthemum	nucleotide	CHS cDNA
29	*chrysCHS*.aa	chrysanthemum	amino acid	*translation of CHS cDNA*
30	*chrysCHS* promoter	chrysanthemum	nucleotide	promoter fragment
31	*LBG*.nt	*Lavandula nil*	nucleotide	*F3'5'H cDNA*
32	*LBG*.aa	*Lavandula nil*	amino acid	*translation of F3'5'H cDNA*

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are schematic representations of the biosynthesis pathway for the flavonoid pigments. Figure 1A illustrates the general production of the anthocyanidin 3-glucosides that occur in most plants that produce anthocyanins. Figure 1B represents further modifications of anthocyanins that occur in petunia. Enzymes involved in the pathway have been indicated as follows: PAL = Phenylalanine ammonia-lyase; C4H = Cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS = Chalcone synthase; CHI = Chalcone flavanone isomerase; F3H = Flavanone 3-hydroxylase; DFR = Dihydroflavonol-4-reductase; ANS = Anthocyanidin synthase, 3GT = UDP-glucose: flavonoid 3-*O*-glucosyltransferase; 3RT = UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase, AR-AT = Anthocyanidin-rutinoside acyltransferase, 5GT = Anthocyanin 5-glucosyltransferase; 3' OMT = Anthocyanin 3' *O*-methyltransferase, 3'5' OMT = Anthocyanin 3', 5' *O* -methyltransferase. Other abbreviations include: DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin,

TABLE 2: Descriptions of the abbreviations used in Figures 2 to 52

ABBREVIATION	DESCRIPTION
Amp	ampicillin resistance gene which confers resistance to the antibiotic ampicillin
ColE1ori	plasmid origin of replication
f1 ori (+)	f1 filamentous phage origin of replication
GentR	gentamycin resistance gene which confers resistance to the antibiotic gentamycin
LB	left border of the T-DNA
nptIII	the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin
ori pRi	plasmid origin of replication
ori 322	plasmid origin of replication
pACYC ori	modified replicon from pACYC184 from *E. coli*

pVS1	a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa*
rev	approximate location of the M13 reverse primer site used in sequence analysis
RB	right border of the T-DNA
TetR	tetracycline resistance gene which confers resistance to the antibiotic tetracycline
-20	approximate location of the M13 -20 primer site used in sequence analysis
RK2	broad host range Gram-negative plasmid RK2 origin

Figure 2 is a diagrammatic representation of the plasmid pCGP602, pCGP601 and pCGP176 containing petunia *F3'5'H petHf1* cDNA clones from *P. hybrida cv. OGB*. The petunia *F3'5'H petHf1* fragment was used in the preparation of constructs containing the petunia F3'5'H cDNA clone. ^{32}P-labelled fragments of the 1.6 kb *Bsp*HI/*Fsp*I fragment were used to probe petal cDNA libraries. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 3 is a diagrammatic representation of the plasmid pCGP175 containing the petunia *F3'5'H petHf2* cDNA clone from *P. hybrida cv. OGB*. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 4 is a diagrammatic representation of the plasmid pCGP1303 containing a subclone of the petunia *F3'5'H petHf1* cDNA clone from pCGP601. The construction of pCGP1303 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 5 is a diagrammatic representation of the binary plasmid pCGP1452. The *AmCHS 5': petHf1: petD8 3'* gene from pCGP485 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1452 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 6 is a diagrammatic representation of the binary plasmid pWTT2132 (DNAP) containing the 35S 5': SuRB selectable marker gene and a multi-cloning site. A description of pWTT2132 is given in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 7 is a diagrammatic representation of the plasmid pCGP725. The *AmCHS 5': petHf1: petD8 3'* gene from pCGP485 was cloned into pBluescript II (KS (+) vector. The construction of pCGP725 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 8 is a diagrammatic representation of the binary plasmid pCGP1453. The *Mac: petHf1: mas 3'* gene from pCGP628 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1453 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 9 is a diagrammatic representation of the binary plasmid pCGP1457. The *petD8 5': petHf1: petD8 3'* gene from pCGP1107 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1457 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 10 is a diagrammatic representation of the binary plasmid pCGP1461. The *shortpetFLS 5': petHf1: petFLS 3'* gene from pCGP497 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1461 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 11 is a diagrammatic representation of the binary plasmid pCGP1616. The *petRT 5': petHf1: nos 3'* gene from pCGP846 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of

pCGP1616 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 12 is a diagrammatic representation of the binary plasmid pCGP1623. The *mas/35S: petHf1: ocs 3'* gene from pCGP1619 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1623 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 13 is a diagrammatic representation of the binary plasmid pCGP1638. The *CaMV 35S: petHf1: nos 3'* gene from pCGP1636 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1636 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 14 is a diagrammatic representation of the binary plasmid pCGP1860. The *RoseCHS 5': petHf1: nos 3'* gene from pCGP200 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1860 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 15 is a diagrammatic representation of the binary plasmid pCGP2123. The *CaMV35S: petHf2: ocs 3'* gene from pCGP2109 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2123 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 16 is a diagrammatic representation of the binary plasmid pCGP1988. The multi-cloning site of the binary vector pWTT2132 (DNAP) was replaced with the multi-cloning site from pNEB193 (New England Biolabs). The construction of pCGP1988 is described in

Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 17 is a diagrammatic representation of the plasmid pCGP2105. The *35S 5': ocs 3'* expression cassette with multiple restriction endonuclease sites between the promoter and terminator fragments is in a pBluescript SK (+) vector backbone. The construction of pCGP2105 is described in Example 4. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 18 is a diagrammatic representation of the binary plasmid pCGP1307. The *petD8 5': GUS: petD8 3'* gene from pCGP1106 was cloned into the binary vector pCGN1548 in a tandem orientation to the chimaeric *nptII* selectable marker gene. The construction of pCGP1307 is described in Example 6. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 19 is a diagrammatic representation of the binary plasmid pCGP1506. The *longpetFLS 5': GUS: petFLS 3'* gene from pCGP496 was cloned into the binary vector pBIN19 in a tandem orientation to the chimaeric *nptII* selectable marker gene. The construction of pCGP1506 is described in Example 6. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 20 is a diagrammatic representation of the binary plasmid pCGP1626. The *ChrysCHS 5': GUS: nos 3'* gene from pCGP1622 was cloned into the binary vector pWTT2132 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1626 is described in Example 6. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 21 is a diagrammatic representation of the binary plasmid pCGP1641. The *petRT 5': GUS: petRT 3'* gene from pCGP1628 was cloned into the binary vector pWTT2132 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1641 is

described in Example 6. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 22 is a diagrammatic representation of the binary plasmid pCGP1861. The *RoseCHS 5': GUS: nos 3'* gene from pCGP197 was cloned into the binary vector pWTT2132 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1861 is described in Example 6. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 23 is a diagrammatic representation of the binary plasmid pCGP1953. The *AmCHS 5': GUS: petD8 3'* gene from pCGP1952 was cloned into the binary vector pWTT2132 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1953 is described in Example 6. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 24 is a diagrammatic representation of the binary plasmid pWTT2084 (DNAP) containing a *35S 5': GUS: ocs 3'* gene in a convergent orientation to the chimaeric *SuRB* selectable marker gene. A description of pWTT2084 is given in Example 6. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 25 is a diagrammatic representation of the plasmid pCGP1959 containing the *F3'5'H BP#18* cDNA clone from *Viola spp.* cv Black Pansy in a pBluescript SK II (+) backbone. A description of pCGP1959 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 26 is a diagrammatic representation of the plasmid pCGP1961 containing the *F3'5'H BP#40* cDNA clone from *Viola spp.* cv Black Pansy in a pBluescript SK II (+) backbone. A description of pCGP1961 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 27 is a diagrammatic representation of the binary plasmid pCGP1972. The *AmCHS 5': BP#18: petD8 3'* gene from pCGP1970 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1972 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 28 is a diagrammatic representation of the binary plasmid pCGP1973. The *AmCHS 5': BP#40: petD8 3'* gene from pCGP1971 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1973 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 29 is a diagrammatic representation of the binary plasmid pCGP1967. The *CaMV 35S: BP#18:ocs 3'* gene from pCGP1965 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1967 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 30 is a diagrammatic representation of the binary plasmid pCGP1969. The *CaMV 35S: BP#40:ocs 3'* gene from pCGP1966 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1969 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 31 is a diagrammatic representation of the plasmid pCGP1995 containing the *F3'5'H Sal#2* cDNA clone from *Salvia spp.* in a pBluescript SK II (+) backbone. A description of pCGP1995 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 32 is a diagrammatic representation of the plasmid pCGP1999 containing the *F3'5'H Sal#47* cDNA clone from *Salvia spp* in a pBluescript SK II (+) backbone. A description of pCGP1999 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 33 is a diagrammatic representation of the binary plasmid pCGP2121. The *AmCHS 5': Sal#2: petD8 3'* gene from pCGP2116 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2121 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 34 is a diagrammatic representation of the binary plasmid pCGP2122. The *AmCHS 5': Sal#47: petD8 3'* gene from pCGP2117 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2122 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 35 is a diagrammatic representation of the binary plasmid pCGP2120. The *CaMV 35S:Sal#2:ocs 3'* gene from pCGP2112 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2120 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 36 is a diagrammatic representation of the binary plasmid pCGP2119. The *CaMV 35S:Sal#47:ocs 3'* gene from pCGP2111 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2119 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 37 is a diagrammatic representation of the plasmid pCGP2110 containing the *F3'5'H Soll#5* cDNA clone from *Sollya spp.* in a pBluescript SK II (+) backbone. A description of pCGP2110 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 38 is a diagrammatic representation of the binary plasmid pCGP2130. The *AmCHS 5': Soll#5: petD8 3'* gene from pCGP2128 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2130 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 39 is a diagrammatic representation of the binary plasmid pCGP2131. The *CaMV 35S: Soll#5:ocs 3'* gene from pCGP2129 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2131 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 40 is a diagrammatic representation of the plasmid pCGP2231 containing the *F3'5'H Kenn#31* cDNA clone from *Kennedia spp.* in a pBluescript SK II (+) backbone. A description of pCGP2231 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 41 is a diagrammatic representation of the binary plasmid pCGP2256. The *AmCHS 5': Kenn#31: petD8 3'* gene from pCGP2242 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2256 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 42 is a diagrammatic representation of the binary plasmid pCGP2252. The *CaMV 35S: Kenn#31:ocs 3'* gene from pCGP2236 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2252 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 43 is a diagrammatic representation of the plasmid pBHF2F containing the full-length *F3'5'H BpeaHF2* cDNA clone from *Clitoria ternatea* in a pBluescript SK II (+) backbone. A description of pBHF2F is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 44 is a diagrammatic representation of the binary plasmid pCGP2135. The *AmCHS 5': BpeaHF2: petD8 3'* gene from pCGP2133 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2135 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 45 is a diagrammatic representation of the binary plasmid pBEBF5. The *eCaMV 35S: BpeaHF2: nos 3'* gene was constructed by replacing the *GUS* fragment from pBE2113-GUSs with the *Clitoria F3'5'H BpeaHF2* cDNA clone from pBHF2F The construction of pBEBF5 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 46 is a diagrammatic representation of the binary plasmid pCGP2134. The *CaMV 35S: BpeaHF2: ocs 3'* gene from pCGP2132 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP2134 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 47 is a diagrammatic representation of the plasmid pG48 containing the *F3'5'H Gen#48* cDNA clone from *Gentiana triflora* in a pBluescript SK II (+) backbone. A description of pG48 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 48 is a diagrammatic representation of the binary plasmid pCGP1498. The *AmCHS 5': Gen#48: petD8 3'* gene from pCGP1496 was cloned into the binary vector pCGP1988 in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1498 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 49 is a diagrammatic representation of the binary plasmid pBEGHF48. The *eCaMV 35S: Gen#48: nos 3'* gene was constructed by replacing the *GUS* fragment from pBE2113-GUSs with the *Gentiana F3'5'H Gen#48* cDNA clone from pG48. The construction of pBEGHF48 is described in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 50 is a diagrammatic representation of the binary plasmid pCGP1982. The *CaMV 35S:Gen#48:ocs 3'* gene from pCGP1981 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. The construction of pCGP1982 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 51 is a diagrammatic representation of the plasmid pLFH8 containing the *F3'5'H LBG* cDNA clone from *Lavandula nil* in a pBluescript SK II (+) backbone. A description of pLFH8 is given in Example 7. Selected restriction endonuclease sites are marked. Refer to Table 2 and Table 4 for a description of the abbreviations.

Figure 52 is a diagrammatic representation of the binary plasmid pBELF8. The *eCaMV 35S: LBG: nos 3'* gene was constructed by replacing the *GUS* fragment from pBE2113-GUSs with the *Lavandula F3'5'H LBG* cDNA clone from pLHF8 The construction of pBELF8 is described in Example 7. Refer to Table 2 and Table 4 for a description of the abbreviations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, genetic sequences encoding polypeptides having F3'5'H activity have been identified, cloned and assessed. The recombinant genetic sequences of the present invention permit the modulation of expression of genes encoding this enzyme by, for example, *de novo* expression, over-expression, sense suppression, antisense inhibition, ribozyme, minizyme and DNAzyme activity, RNAi-induction or methylation-induction or other transcriptional or post-transcriptional silencing activities. RNAi-induction includes genetic molecules such as hairpin, short double stranded DNA or RNA, and partially double stranded DNAs or RNAs with one or two single stranded nucleotide over hangs. The ability to control F3'5'H synthesis in plants permits modulation of the composition of individual anthocyanins as well as alteration of relative levels of flavonols and anthocyanins, thereby enabling the manipulation of petal color. Moreover, the present invention extends to plants and reproductive or vegetative parts thereof including flowers, fruits, seeds, vegetables, leaves, stems and the like. The present invention further extends to ornamental transgenic or genetically modified plants. The term "transgenic" also includes progeny plants and plants from subsequent genetics and/or crosses thereof from the primary transgenic plants.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in detectable levels of delphinidin or delphinidin-based molecules as measured by a chromatographic technique.

Another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in a sufficient level and length of transcript which is translated to said F3'5'H as determined by detectable levels of delphinidin or delphinidin-based molecules as measured by a chromatographic technique.

A further aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results a full-length transcript which is detectable by Northern blot analysis of total RNA isolated from rose petals.

The present invention is described and exemplified herein by reference to the identification, cloning and manipulation of genetic sequences encoding a F3'5'H which acts on DHK as well as DHQ. Preferably, the F3'5'H enzyme is a pansy, salvia , sollya lavender or kennedia F3'5'H. The F3'5'H enzyme may also be considered to include a polypeptide or protein having a F3'5'H activity or F3'5'H-like activity. The latter encompasses derivatives having altered F3'5'H activities.

A preferred aspect of the present invention, therefore, is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding a F3'5'H or a functional mutant, derivative, part, fragment, homolog or analog thereof wherein the nucleic acid molecule is characterized by the following:

(i) the F3'5'H transcript in rose petal tissue is of sufficient level and size to encode a F3'5'H resulting in detectable delphinidin or delphinidin-based molecules in the rose petal tissue as measured by a chromatographic procedure (eg. TLC or HPLC);

(ii) the *F3'5'H* transcript in rose petal tissue is full-length and detected by Northern blot analysis of total RNA isolated from rose petal tissue

(iii) the F3'5'H in rose petal tissue results in detectable delphinidin or delphinidin-based molecules as measured by a chromatographic procedure (eg. TLC or HOPLC); and/or

(iv) the F3'5'H results in a visual color change in rose petal tissue.

The term delphinidin-based pigments includes the anthocyanidin, delphinidin or any derivatives thereof including but not limited to glycosylated, acylated, methylated or other modified forms. Methylated forms of delphinidin include but are not limited to the anthocyanidin petunidin (methylated at the 3'-position), malvidin (methylated at the 3' and 5' position), 5-O methyl malvidin (methylated at the 5, 3' and 5' positions), 5, 7-O dimethyl malvidin (methylated at the 5, 7, 3' and 5' positions). The methylated anthocyanidins can also be modified by glycosylation and acylation. The term anthocyanins defines glycosylated forms of the respective anthocyanidins.

By the term "nucleic acid molecule" is meant a genetic sequence in a non-naturally occurring condition. Generally, this means isolated away from its natural state or synthesized or derived in a non-naturally-occurring environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids. It also extends to the genomic DNA or cDNA or part thereof encoding F3'5'H or a part thereof in reverse orientation relative to its own or another promoter. It further extends to naturally occurring sequences following at least a partial purification relative to other nucleic acid sequences.

The term "genetic sequences" is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or *via* a complementary series of bases, a sequence of amino acids in a F3'5'H enzyme. Such a sequence of amino acids may constitute a full-length F3'5'H such as is set forth in SEQ ID NO: 10 (pansy) or SEQ ID NO:12 (pansy) or SEQ ID NO:14 (salvia) or SEQ ID NO:16 (salvia) or SEQ ID NO:18 (sollya) or SEQ ID NO:32 (lavender) or SEQ ID NO:27 (kennedia) or an active truncated form thereof or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme. A genetic sequence may also be referred to as a sequence of nucleotides or a nucleotide sequence and includes a recombinant fusion of two or more sequences.

In accordance with the above aspects of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or SEQ ID NO:31 (lavender) or SEQ ID NO:26 (kennedia) or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 under low stringency conditions.

Table 1 provides a summary of the sequence identifiers.

Alternative percentage similarities and identities (at the nucleotide or amino acid level) encompassed by the present invention include at least about 60% or at least about 65% or at least about 70% or at least about 75% or at least about 80% or at least about 85% or at least about 90% or above, such as about 95% or about 96% or about 97% or about 98% or about 99%, such as at least about 60%, 61%, 62%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%.

In a particularly preferred embodiment, there is provided an isolated nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or SEQ ID NO:31 (lavender) or SEQ ID NO:26 (kennedia) or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID NO:1 (petunia) or SEQ ID NO:3 (petunia) or complementary strands of either under low stringency conditions, wherein said nucleotide sequence encodes a polypeptide having a F3'5'H activity.

For the purposes of determining the level of stringency to define nucleic acid molecules capable of hybridizing to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:9 or SEQ ID NO:11

or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 reference herein to a low stringency includes and encompasses from at least about 0% to at least about 15% v/v formamide and from at least about 1M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace the inclusion of formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J. Mol. Biol. 5:* 109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem. 46:* 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 1.0% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 1.0% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or SEQ ID NO:32 or SEQ ID NO:27 or an amino acid sequence having at least about 50% similarity thereto.

The term similarity as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level,

similarity includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, similarity includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res. 25:* 3389-3402, 1997). A detailed discussion of sequence analysis can be found in Unit

19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity.

The nucleic acid molecules of the present invention may be further characterized by having, or previously having, prior to derivatization on overall lower AT content (or higher GC content) compared to a nucleic acid molecule which encodes a F3'5'H but which does not result in detectable intact transcript in rose petal tissue or, when expressed, does not result in detectable delphinidin or delphinidin-based molecules, as measured by a chromatographic procedure such as TLC or HPLC. Furthermore, the % of A's or T's in the third position of a codon is also lower than other F4'5'H enzymes. Reference herein to a chromatographic procedure includes a related procedure. By "related" means a technically related procedure or a procedure which provides a similar result. Examples of related procedures include other forms of chromatography (eg. gas chromatography).

In addition, nucleotide sequences which do not express well in rose tissue may be modified such as in reducing overall % AT or at least reduce the levels of % AT in the third position of a codon. Such time expression in rose tissue is elevated.

The nucleic acid sequences contemplated herein also encompass oligonucleotides useful as genetic probes for amplification reactions or as antisense or sense molecules capable of regulating expression of the corresponding gene in a plant. Sense molecules include hairpin constructs, short double stranded DNAs and RNAs and partially double stranded DNAs and RNAs which one or more single stranded nucleotide over hangs. An antisense molecule as used herein may also encompass a genetic construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its or another promoter. It may also encompass a homologous genetic sequence. An antisense or sense molecule may also be directed to terminal or internal portions of the gene encoding a polypeptide having a F3'5'H activity or to combinations of the above such that the expression of the gene is reduced or eliminated.

With respect to this aspect of the invention, there is provided an oligonucleotide of 5-50 nucleotides such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55 having substantial similarity to a part or region of a molecule with a nucleotide sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26. By substantial similarity or complementarity in this context is meant a hybridizable similarity under low, alternatively and preferably medium and alternatively and most preferably high stringency conditions specific for oligonucleotide hybridization (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, 1989). Such an oligonucleotide is useful, for example, in screening for F3'5'H genetic sequences from various sources or for monitoring an introduced genetic sequence in a transgenic plant. The preferred oligonucleotide is directed to a conserved F3'5'H genetic sequence or a sequence conserved within a plant genus, plant species and/or plant variety.

In one aspect of the present invention, the oligonucleotide corresponds to the 5' or the 3' end of the F3'5'H genetic sequences. For convenience, the 5' end is considered herein to define a region substantially between the start codon of the structural gene to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural gene. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

In one embodiment, the nucleic acid sequence encoding a F3'5'H or various functional derivatives thereof is used to reduce the level of an endogenous a F3'5'H (e.g. *via* co-suppression or antisense-mediated suppression) or other post-transcriptional gene silencing (PTGS) processes including RNAi or alternatively the nucleic acid sequence encoding this enzyme or various derivatives or parts thereof is used in the sense or antisense orientation to reduce the level of a F3'5'H. The use of sense strands, double or partially single stranded such as constructs with hairpin loops is particularly useful in inducing a PTGS response. In a further alternative, ribozymes, minizymes or DNAzymes could be used to inactivate target nucleic acid sequences.

Still a further embodiment encompasses post-transcriptional inhibition to reduce translation into polypeptide material. Still yet another embodiment involves specifically inducing or removing methylation.

Reference herein to the altering of a F3'5'H activity relates to an elevation or reduction in activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. Such elevation or reduction may be referred to as modulation of a F3'5'H enzyme activity. Generally, modulation is at the level of transcription or translation of F3'5'H genetic sequences.

The nucleic acids of the present invention may be a ribonucleic acid or deoxyribonucleic acids, single or double stranded and linear or covalently closed circular molecules. Preferably, the nucleic acid molecule is cDNA. The present invention also extends to other nucleic acid molecules which hybridize under low, preferably under medium and most preferably under high stringency conditions with the nucleic acid molecules of the present invention and in particular to the sequence of nucleotides set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 or a part or region thereof. In its most preferred embodiment, the present invention extends to a nucleic acid molecule having a nucleotide sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 or to a molecule having at least 40%, more preferably at least 45%, even more preferably at least 55%, still more preferably at least 65%-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of the sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26 and wherein the nucleic acid encodes or is complementary to a sequence which encodes an enzyme having a F3'5'H activity. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and yet still encode a F3'5'H activity and such molecules may still be considered in the scope of the present invention where they have regions of sequence conservation. The present invention further extends to nucleic acid molecules in the form of oligonucleotide primers or probes capable of hybridizing to a portion of the nucleic acid molecules contemplated above, and in particular those set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or SEQ ID NO:31 or SEQ ID NO:26, under low, preferably under medium and most preferably under high stringency conditions. Preferably the portion corresponds to the 5' or the 3' end of the gene. For convenience the 5' end is considered herein to define a region substantially between the start codon of the structural genetic sequence to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural genetic sequence. It is clear, therefore, that

oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

The term gene is used in its broadest sense and includes cDNA corresponding to the exons of a gene. Accordingly, reference herein to a gene is to be taken to include:-

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term gene is also used to describe synthetic or fusion molecules encoding all or part of an expression product. In particular embodiments, the term nucleic acid molecule and gene may be used interchangeably.

The nucleic acid or its complementary form may encode the full-length enzyme or a part or derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally occurring enzyme and which retains a F3'5'H activity. In this regard, the nucleic acid includes the naturally occurring nucleotide sequence encoding a F3'5'H or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally occurring sequence. The nucleic acid of the present invention or its complementary form may also encode a "part" of the F3'5'H, whether active or inactive, and such a nucleic acid molecule may be useful as an oligonucleotide probe, primer for polymerase chain reactions or in various mutagenic techniques, or for the generation of antisense molecules.

Reference herein to a "part" of a nucleic acid molecule, nucleotide sequence or amino acid sequence, preferably relates to a molecule which contains at least about 10 contiguous nucleotides or five contiguous amino acids, as appropriate.

Amino acid insertional derivatives of the F3'5'H of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 3.

TABLE 3 Suitable residues for amino acid substitutions

Original residue	Exemplary substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn; Glu
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile; Val
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser

Original residue	Exemplary substitutions
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu; Met

Where the F3'5'H is derivatized by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, *J. Am. Chem. Soc. 85:* 2149, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.* (1989, *supra*).

Other examples of recombinant or synthetic mutants and derivatives of the F3'5'H enzyme of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogs" and "derivatives" also extend to any functional chemical equivalent of a F3'5'H and also to any amino acid derivative described above. For convenience, reference to F3'5'H herein includes reference to any functional mutant, derivative, part, fragment, homolog or analog thereof.

The present invention is exemplified using nucleic acid sequences derived from pansy, salvia, sollya or lavender or kennedia since this represents the most convenient and preferred source of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms. All such nucleic acid sequences encoding directly or indirectly a F3'5'H are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding F3'5'H include, but are not limited to *Vitis spp.*, *Babiana stricta*, *Pinus spp.*, *Picea spp.*, *Larix spp.*, *Phaseolus spp.*, *Vaccinium spp.*, *Cyclamen spp.*, *Iris spp.*, *Pelargonium spp.*; Liparieae, *Geranium spp.*, *Pisum spp.*, *Lathyrus spp.*, *Clitoria spp.*, *Catharanthus spp.*, *Malva spp.*, *Mucuna spp.*, *Vicia spp.*, *Saintpaulia spp.*, *Lagerstroemia spp.*, *bouchina spp.*, *Plumbago spp.*, *Hypocalyptus spp.*, *Rhododendron spp.*, *Linum spp.*, *Macroptilium spp.*, *Hibiscus spp.*, *Hydrangea spp.*, *Cymbidium spp.*, *Milletia spp.*, *Hedysarum spp.*, *Lespedeza spp.*, *Asparagus spp.* *Antigonon spp.*, *Freesia spp.*, *Brunella spp.*, *Clarkia spp.*, etc.

In accordance with the present invention, a nucleic acid sequence encoding a F3'5'H may be introduced into and expressed in a transgenic plant in either orientation thereby providing a means either to convert suitable substrates, if synthesized in the plant cell, ultimately into DHM, or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing F3'5'H activity. The production of these 3', 5'-hydroxylated substrates will subsequently be converted to delphinidin-based pigments that will modify petal color and may contribute to the production of a bluer color. Expression of the nucleic acid sequence in the plant may be constitutive, inducible or developmental and may also be tissue-specific. The word "expression" is used in its broadest sense to include production of RNA or of both RNA and protein. It also extends to partial expression of a nucleic acid molecule.

According to this aspect of the present invention, there is provided a method for producing a transgenic flowering plant capable of synthesizing a F3'5'H, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said F3'5'H under conditions permitting the eventual

expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous F3'5'H at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing flavonoid 3', 5'-hydroxylase activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing F3'5'H activity, said method comprising altering the *F3'5'H* gene through modification of the indigenous sequences via homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

As used herein an "indigenous" enzyme is one, which is native to or naturally expressed in a particular cell. A "non-indigenous" enzyme is an enzyme not native to the cell but expressed through the introduction of genetic material into a plant cell, for example, through a transgene. An "endogenous" enzyme is an enzyme produced by a cell but which may or may not be indigenous to that cell.

In a preferred embodiment, the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered floral or inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic

plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

Alternatively, said method may comprise stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the indigenous or existing F3'5'H. Preferably the altered level would be less than the indigenous or existing level of F3'5'H activity in a comparable non-transgenic plant. Without wishing to limit the present invention, one theory of mode of action is that reduction of the indigenous F3'5'H activity requires the expression of the introduced nucleic acid sequence or its complementary sequence. However, expression of the introduced genetic sequence or its complement may not be required to achieve the desired effect: namely, a flowering plant exhibiting altered floral or inflorescence properties.

In a related embodiment, the present invention contemplates a method for producing a flowering plant exhibiting altered floral or inflorescence properties, said method comprising alteration of the flavonoid 3', 5'-hydroxylase gene through modification of the indigenous sequences via homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Preferably, the altered floral or inflorescence includes the production of different shades of blue or purple or red flowers or other colors, depending on the genotype and physiological conditions of the recipient plant.

Accordingly, the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding a F3'5'H or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule encoding the F3'5'H, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of

nucleotides encoding, or complementary to a sequence encoding, a F3'5'H, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell. By suitable plant is meant a plant capable of producing DHK and possessing the appropriate physiological properties required for the development of the color desired.

One skilled in the art will immediately recognise the variations applicable to the methods of the present invention, such as increasing or decreasing the expression of the enzyme naturally present in a target plant leading to differing shades of colors such as different shades of blue, purple or red.

The present invention, therefore, extends to all transgenic plants or parts or cells therefrom of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered floral or inflorescence properties. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding a F3'5'H. Generally, the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of a F3'5'H nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to seeds from such transgenic plants. Such seeds, especially if colored, are useful as proprietary tags for plants. Any and all methods for introducing genetic material into plant cells including but not limited to *Agrobacterium*-mediated transformation, biolistic particle bombardment etc. are encompassed by the present invention.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts or cells therefrom of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in particular, the extracts from those transgenic plants when used as a flavouring or food additive or health product or beverage or juice or coloring.

Plant parts contemplated by the present invention includes, but is not limited to flowers, fruits, vegetables, nuts, roots, stems, leaves or seeds.

The extracts of the present invention may be derived from the plants or plant part or cells therefrom in a number of different ways including but not limited to chemical extraction or heat extraction or filtration or squeezing or pulverization.

The plant, plant part or cells therefrom or extract can be utilized in any number of different ways such as for the production of a flavouring (e.g. a food essence), a food additive (e.g. a stabilizer, a colorant) a health product (e.g. an antioxidant, a tablet) a beverage (e.g. wine, spirit, tea) or a juice (e.g. fruit juice) or coloring (e.g. food coloring, fabric coloring, dye, paint, tint).

A further aspect of the present invention is directed to recombinant forms of F3'5'H. The recombinant forms of the enzyme will provide a source of material for research, for example, more active enzymes and may be useful in developing *in vitro* systems for production of colored compounds.

Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing a F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

The term genetic construct has been used interchangably throughout the specification and alims with the terms "fusion molecule", "recombinant molecule", "recombinant nucleotide sequence". A genetic construct may include a single nucleic acid molecule comprising a nucleotide sequence encoding a singal protein or may contain multiple open reading frames encoding 2 or more proteins. It may also contain a promoter operably linked to 1 or more of the open reading frames.

Another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding a F3'5'H extrachromasomally in plasmid form.

The present invention further extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or SEQ ID NO:32 or SEQ ID NO:27 or an amino acid sequence having at least about 50% similarity to SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or SEQ ID NO:32 or SEQ ID NO:27 or a derivative of said polypeptide.

A "recombinant polypeptide" means a polypeptide encoded by a nucleotide sequence introduced into a cell directly or indirectly by human intervention or into a parent or other relative or precursor of the cell. A recombinant polypeptide may also be made using cell-free, *in vitro* transcription systems. The term "recombinant polypeptide" includes an isolated polypeptide or when present in a cell or cell preparation. It may also be in a plant or parts of a plant regenerated from a cell which produces said polypeptide.

A "polypeptide" includes a peptide or protein and is encompassed by the term "enzyme".

The recombinant polypeptide may also be a fusion molecule comprising two or more heterologous amino acid sequences.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

General methods

In general, the methods followed were as described in Sambrook *et al.* (1989, *supra*) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual 3rd edition, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, 2001 or Plant Molecular Biology Manual (2nd edition), Gelvin and Schilperoot (eds), Kluwer Academic Publisher, The Netherlands, 1994 or Plant Molecular Biology Labfax, Croy (ed), Bios scientific Publishers, Oxford, UK, 1993.

The cloning vectors pBluescript and PCR script were obtained from Stratagene, USA. pCR7 2.1 was obtained from Invitrogen, USA.

E. coli transformation

The *Escherichia coli* strains used were:

DH5α

supB44, Δ (lacZYA-ArgF)U169, (ø80lacZΔM15), hsdR17(r_k^-, m_k^+),
recA1, endA1, gyrA96, thi-1, relA1, deoR. (Hanahan, *J. Mol. Biol. 166:* 557, 1983)

XL1-Blue

supB44, hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1,
lac^-,[F'proAB, $lacI^q$, lacZΔM15, Tn10(tet^R)] (Bullock *et al., Biotechniques 5:* 376, 1987).

BL21-CodonPlus-RIL strain

ompT hsdS(Rb- mB-) *dcm*+ Tet^r *gal endA* Hte [*argU ileY leuW* Cam^r]

M15 *E. coli* is derived from *E.coli* K12 and has the phenotype Nal^s, Str^s, Rif^s, Thi^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+.

Transformation of the *E. coli* strains was performed according to the method of Inoue *et al.*, (*Gene 96:* 23-28, 1990).

***Agrobacterium tumefaciens* strains and transformations**

The disarmed *Agrobacterium tumefaciens* strain used was AGL0 (Lazo *et al.* *Bio/technology 9:* 963-967, 1991).

Plasmid DNA was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL LB culture (Sambrook *et al.*, 1989, *supra*) and incubation for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% (v/v) 100 mM $CaCl_2$/15% (v/v) glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells were then mixed with 1mL of LB (Sambrook *et al.*, 1989 *supra*) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying the plasmid were selected on LB agar plates containing appropriate antibiotics such as 50 μg/mL tetracycline or 100 μg/mL gentamycin. The confirmation of the plasmid in *A. tumefaciens* was done by restriction endonuclease mapping of DNA isolated from the antibiotic-resistant transformants.

DNA ligations

DNA ligations were carried out using the Amersham Ligation Kit or Promega Ligation Kit according to procedures recommended by the manufacturer.

Isolation and purification of DNA fragments

Fragments were generally isolated on a 1% (w/v) agarose gel and purified using the QIAEX II Gel Extraction kit (Qiagen) or Bresaclean Kit (Bresatec, Australia) following procedures recommended by the manufacturer.

Repair of overhanging ends after restriction endonuclease digestion

Overhanging 5' ends were repaired using DNA polymerase (Klenow fragment) according to standard protocols (Sambrook *et al.*, 1989 *supra*). Overhanging 3' ends were repaired using T4 DNA polymerase according to standard protocols (Sambrook *et al.*, 1989 *supra*).

Removal of phosphoryl groups from nucleic acids

Shrimp alkaline phosphatase (SAP) (USB) was typically used to remove phosphoryl groups from cloning vectors to prevent re-circularization according to the manufacturer's recommendations.

Polymerase Chain Reaction (PCR)

Unless otherwise specified, PCR conditions using plasmid DNA as template included using 2 ng of plasmid DNA, 100 ng of each primer, 2 µL 10 mM dNTP mix, 5 µL 10 x Taq DNA polymerase buffer, 0.5 µL Taq DNA Polymerase in a total volume of 50 µL. Cycling conditions comprised an initial denaturation step of 5 minutes at 94°C, followed by 35 cycles of 94°C for 20 sec, 50°C for 30 sec and 72°C for 1 minute with a final treatment at 72°C for 10 minutes before storage at 4°C.

PCRs were performed in a Perkin Elmer GeneAmp PCR System 9600.

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 µCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on Sephadex G-50 (Fine) columns or Microbiospin P-30 Tris chromatography columns (BioRad).

Plasmid Isolation

Single colonies were analyzed for inserts by inoculating LB broth (Sambrook *et al.*, 1989, *supra*) with appropriate antibiotic selection (e.g. 100 µg/mL ampicillin or 10 to 50 µg/mL tetracycline etc.) and incubating the liquid culture at 37°C (for *E. coli*) or 29°C (for *A. tumefaciens*) for ~16 hours with shaking. Plasmid DNA was purified using the alkali-lysis procedure (Sambrook *et al.*, 1989, *supra*) or using The WizardPlus SV minipreps DNA purification system (Promega) or Qiagen Plasmid Mini Kit (Qiagen). Once the presence of an insert had been determined, larger amounts of plasmid DNA were prepared from 50 mL overnight cultures using the alkali-lysis procedure (Sambrook *et al.*, 1989, *supra*) or

QIAfilter Plasmid Midi kit (Qiagen) and following conditions recommended by the manufacturer.

DNA Sequence Analysis

DNA sequencing was performed using the PRISM (trademark) Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). Sequencing runs were generally performed by the Australian Genome Research Facility at The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) or in-house on an automated 373A DNA sequencer (Applied Biosystems).

Sequences were analysed using a MacVector™ application (version 6.5.3) (Oxford Molecular Ltd., Oxford, England).

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85(8):* 2444-2448, 1988) or BLAST programs (Altschul *et al., J. Mol. Biol. 215(3):* 403-410, 1990). Percentage sequence similarities were obtained using LALIGN program (Huang and Miller, *Adv. Appl. Math. 12:* 373-381, 1991) or ClustalW program (Thompson *et al., Nucleic Acids Research 22:* 4673-4680, 1994) within the MacVector™ application (Oxford Molecular Ltd., England) using default settings.

Multiple sequence alignments were produced using ClustalW (Thompson *et al.,* 1994, *supra*) using default settings.

EXAMPLE 2

Plant transformations

Petunia hybrida transformations (Sw63 x Skr4)

As described in Holton *et al.* (1993a, *supra*) by any other method well known in the art.

Rosa hybrida transformations

As described in U.S. Patent Application No. 542,841 (PCT/US91/04412) or Robinson and Firoozabady (*Scientia Horticulturae, 55*: 83-99, 1993), Rout *et al.* (*Scientia Horticulturae, 81*: 201-238, 1999) or Marchant *et al.* (*Molecular Breeding 4*: 187-194, 1998) or by any other method well known in the art.

Cuttings of *Rosa hybrida* were generally obtained from Van Wyk and Son Flower Supply, Victoria.

Dianthus caryophyllus transformations

International Patent Application No. PCT/US92/02612 (carnation transformation). As described in International Patent Application No. PCT/AU96/00296 (Violet carnation), Lu *et al.* (*Bio/Technology 9*: 864-868, 1991), Robinson and Firoozabady (1993, *supra*) or by any other method well known in the art.

Cuttings of *Dianthus caryophyllus* cv. Kortina Chanel or Monte Lisa were obtained from Van Wyk and Son Flower Supply, Victoria.

EXAMPLE 3

Transgenic Analysis

Color coding

The Royal Horticultural Society's Color Chart (Kew, UK) was used to provide a description of observed color. They provide an alternative means by which to describe the color phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colors and should not be regarded as limiting the possible colors which may be obtained.

Chromatographic analysis

Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analysis was performed generally as described in Brugliera *et al.* (*Plant J.* 5, 81-92, 1994).

Extraction of anthocyanidins

Prior to HPLC analysis, the anthocyanin and flavonol molecules present in petal and stamen extracts were acid hydrolysed to remove glycosyl moieties from the anthocyanidin or flavonol core. Anthocyanidin and flavonol standards were used to help identify the compounds present in the floral extracts.

Anthocyanidins in the reaction mixture were analysed by HPLC *via* gradient elution using gradient conditions of 50%B to 60%B over 10 minutes, then 60% B for 10 minutes and finally 60% B to 100% B over 5 minutes where solvent A consisted of TFA: H_2O (5:995) and solvent B consisted of acetonitrile: TFA: H_2O (500:5:495). An Asahi Pac ODP-50 cartridge column (250 mm x 4.6 mm ID) was used for the reversed phase chromatographic separations. The flow rate was 1 mL/min and the temperature was 40°C. The detection of the anthocyanidin compounds was carried out using a Shimadzu SPD-M6A three dimensional detector at 400-650 nm.

The anthocyanidin peaks were identified by reference to known standards, *viz* delphinidin or delphinidin-based molecules, petunidin, malvidin, cyanidin and peonidin

Stages of flower development

Petunia

Petunia hybrida cv. Skr4 x Sw63 flowers were harvested at developmental stages defined as follows:

Stage 1:	Unpigmented, closed bud.
Stage 2:	Pigmented, closed bud.
Stage 3:	Pigmented bud with emerging corolla
Stage 4:	Pigmented, opened flower with anther intact (pre-dehiscence)
Stage 5:	Fully opened flower with all anthers dehisced.

For TLC or HPLC analysis, petals were collected from stage 4 flowers at the stage of maximum pigment accumulation.

For Northern blot analysis, petals were collected from stages 2 to 3 flowers at the stage of maximal expression of flavonoid pathway genes.

Carnation

Dianthus caryophyllus flowers were harvested at developmental stages defined as follows:

Stage 1:	Closed bud, petals not visible.
Stage 2:	Flower buds opening: tips of petals visible.
Stage 3:	Tips of nearly all petals exposed. "Paint-brush stage".
Stage 4:	Outer petals at 45° angle to stem.
Stage 5:	Flower fully open.

For TLC or HPLC analysis, petals were collected from stage 4 flowers at the stage of maximum pigment accumulation.

For Northern blot analysis, petals were collected from stage 3 flowers at the stage of maximal expression of flavonoid pathway genes.

Rose

Stages of *Rosa hybrida* flower development were defined as follows:

Stage 1: Unpigmented, tightly closed bud.
Stage 2: Pigmented, tightly closed bud
Stage 3: Pigmented, closed bud; sepals just beginning to open.
Stage 4: Flower bud beginning to open; petals heavily pigmented; sepals have separated.
Stage 5: Sepals completely unfolded; some curling. Petals are heavily pigmented and unfolding.

For TLC or HPLC analysis, petals were collected from stage 4 flowers at the stage of maximum pigment accumulation.

For Northern blot analysis, petals were collected from stage 3 to 4 flowers at the stage of maximal expression of flavonoid pathway genes (Tanaka *et al.*, *Plant Cell Physiol.*, 36(6): 1023-1031, 1995).

Anthocyanin/flavonol measurements by spectrophotometric measurements

Approximately 200mg of fresh petal tissue was added to 2 mL of methanol/1% (v/v) HCl and incubated for ~16 hours at 4°C. A 1 in 20 dilution (e.g. 50 µL made to 1000 µL) was then made and the absorbance at 350 nm and 530 nm was recorded.

The approximate flavonols and anthocyanin amounts (nmoles/gram) were then calculated according to the following formulae:

Anthocyanin content

$$\frac{(A_{530}/34{,}000) \times \text{volume of extraction buffer (mL)} \times \text{dilution factor} \times 10^6}{\text{mass of petal tissue (grams)}}$$

Flavonol content

$$\frac{(A_{350}/14{,}300) \times \text{volume of extraction buffer (mL)} \times \text{dilution factor} \times 10^6}{\text{mass of petal tissue (grams)}}$$

Northern/RNA blot analysis

Transcription of a transferred gene was monitored by isolating RNA and estimating the quantity and size of the expected transcript. Northern blot analysis was used to monitor the steady-state level of particular transcripts in petals. A transcript was determined to be intact or full-length based on the estimated size expected from the gene used. In general when cDNAs were used as coding sequences the size of the transcript expected would be the size of the cDNA plus any 5' untranslated component of the fused promoter fragment plus any 3' untranslated sequence from the fused terminator fragment. In some cases where a cDNA region contained a putative polyadenylation site and the terminator region contained a putative polyadenylation site, 2 transcripts would be detected. One would be of a size consistent with polyadenylation occurring just downstream from the polyadenylation site within the cDNA sequence. The second transcript would be larger and consistent with the transcript being polyadenylated after the polyadenylation site within the terminator fragment.

Total RNA was isolated from petals or leaves using a Plant RNAeasy kit (QIAGEN) following procedures recommended by the manufacturer. For rose samples 1% (w/v) PVP was added to the extraction buffer.

RNA samples (5 μg) were electrophoresed through 2.2 M formaldehyde/1.2% w/v agarose gels using running buffer containing 40 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was stained with ethidium bromide and visualised under UV-light. The ribosomal RNA was generally used as a guide in confirming that the RNA had not been degraded by intra- or extra- cellular ribonucleases. The RNA was transferred to Hybond-N membrane filters (Amersham) and treated as described by the manufacturer.

Control samples were included on RNA gels as a measure of the integrity of the radiolabelled probe and as guides to expected transcript sizes. Controls for *petHf1* and *petHf2* genes included RNA isolated from petunia OGB petals (stages 3 to 4) or from flowers of transgenic carnations shown previously to accumulate *petHf1* transcripts. Controls for other *F3'5'H* genes generally included RNA isolated from petals of the same species from which the *F3'5'H* sequence had been isolated.

RNA blots were probed with ^{32}P-labelled fragments. Prehybridization (1 hour at 42°C) and hybridization (16 hours at 42°C) of the membrane filters were carried out in 50% v/v formamide, 1 M NaCl, 1% w/v SDS, 10% w/v dextran sulphate. The membrane filters were generally washed in 2 x SSC, 1% w/v SDS at 65°C for between 1 to 2 hours and then 0.2 x SSC, 1% w/v SDS at 65°C for between 0.5 to 1 hour. Membrane filters were generally exposed to Kodak XAR film with an intensifying screen at -70°C for 16 to 72 hours.

EXAMPLE 4

Introduction of chimeric petunia F3'5'H genes into rose

As described in the introduction, the pattern of hydroxylation of the B-ring of the anthocyanidin molecule plays a key role in determining petal color. The production of the dihydroflavonol DHM, leads to the production of the purple/blue delphinidin-based pigments in plants such as petunia. The absence of the F3'5'H activity has been correlated

with the absence of blue flowers in many plant species such as *Rosa*, *Gerbera*, *Antirrhinum*, *Dianthus* and *Dendranthema*.

Based on success in producing delphinidin-based pigments in a mutant petunia line (Holton *et al.*, 1993a, *supra* and International Patent Application No. PCT/AU92/00334), in tobacco flowers (International Patent Application No. PCT/AU92/00334) and in carnation flowers (International Patent Application No. PCT/AU96/00296), similar chimeric petunia *F3'5'H* genes were also introduced into roses in order to produce novel delphinidin-based pigments and modify flower color.

Preparation of chimeric petunia F3'5'H gene constructs

A summary of promoter, terminator and coding fragments used in the preparation of constructs and the respective abbreviations is listed in Table 4.

TABLE 4 Abbreviations used in construct preparations

ABBREVIATION	DESCRIPTION
AmCHS 5'	1.2 kb promoter fragment from the *Antirrhinum majus* chalcone synthase (*CHS*) gene (Sommer and Saedler, *Mol Gen. Gent.*, 202: 429-434, 1986)
CaMV 35S	~0.2 kb incorporating *BglII* fragment containing the promoter region from the Cauliflower Mosaic Virus 35S (*CaMV 35S*) gene (Franck *et al.*, *Cell* 21: 285-294, 1980, Guilley *et al.*, *Cell*, 30: 763-773. 1982)
35S 5'	promoter fragment from *CaMV 35S* gene (Franck *et al.*, 1980, *supra*) with an ~60bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) (Harpster *et al.*, *MGG*, 212: 182-190, 1988)
chrysCHS 5'	promoter region from a *CHS* gene from chrysanthemum (SEQ ID NO: 30)

ABBREVIATION	DESCRIPTION
aCaMV 35S	enhanced *CaMV 35S* promoter as described in Mitsuhara *et al.*, *Plant Cell Physiol.* *37:* 49-59, 1996
GUS	β-glucuronidase (*GUS*) coding sequence (Jefferson, *et al.*, *EMBO J.* 6: 3901-3907, 1987)
Mac	Hybrid promoter consisting of the promoter from the mannopine synthase (*mas*) gene and a *CaMV 35S* enhancer region (Comai *et al.*, *Plant Mol. Biol.* 15: 373-381, 1990)
mas/35S	Hybrid promoter consisting of a promoter region from *CaMV 35S* gene with enhancer elements from a promoter fragment of mannopine synthase (*mas*) gene of *Agrobacterium tumefaciens* (Janssen and Gardner, *Plant Molecular Biology*, 14: 61-72, 1989)
mas 5'	Promoter region from the *mas* of *A. tumefaciens*
mas 3'	Terminator region from the *mas* gene of *A. tumefaciens*
nos 5'	Promoter region from the nopaline synthase (*nos*) gene of *A. tumefaciens* (Depicker *et al.*, *J Mol. and Appl. Genetics* 1: 561-573, 1982)
nos 3'	Terminator region from the *nos* gene of *A. tumefaciens* (Depicker *et al.*, 1982, *supra*)
nptII	Kanamycin-resistance gene (encodes neomycin phosphotransferase which deactivates aminoglycoside antibiotics such as kanamycin, neomycin and G418)
ocs 3'	~1.6kb terminator fragment from octopine synthase gene of *A. tumefaciens* (described in Janssen and Gardner, 1989, *supra*)
petD8 5'	~3.2kb promoter region from a phospholipid transfer protein gene (*D8*) of *Petunia hybrida* (Holton, Isolation and characterization of petal specific genes from *Petunia hybrida*. PhD thesis, University of Melbourne, Australia, 1992) (SEQ ID NO: 24)
petD8 3'	~0.7kb terminator region from a phospholipid transfer protein gene (*D8*) of *Petunia hybrida* cv. OGB (Holton, 1992, *supra*)

ABBREVIATION	DESCRIPTION
long petFLS 5'	~4.0kb fragment containing the promoter region from a flavonol synthase (*FLS*) gene of *P. hybrida*
short petFLS 5'	~2.2kb fragment containing the promoter region from *FLS* gene of *P. hybrida*
petFLS 3'	~0.95kb fragment containing the terminator region from *FLS* gene of *P. hybrida*
petHf1	Petunia *F3'5'H Hf1* cDNA clone (Holton *et al.*, 1993a, *supra*) (SEQ ID NO: 1)
petHf2	Petunia *F3'5'H Hf2* cDNA clone (Holton *et al.*, 1993a, *supra*) (SEQ ID NO: 3)
petRT 5'	Promoter region of an anthocyanidin-3- glucoside rhamnosyltransferase (*3RT*) gene from *P. hybrida* (Brugliera, Characterization of floral specific genes isolated from *Petunia hybrida*. RMIT, Australia. PhD thesis, 1994)
petRT 3'	Terminator region of a *3RT* gene from *P. hybrida* (Brugliera, 1994, *supra*)
RoseCHS 5'	~2.8kb fragment containing the promoter region from a *CHS* gene of *Rosa hybrida* (SEQ ID: 5)
SuRB	Chlorsulfuron-resistance gene (encodes Acetolactate Synthase) with its own terminator from *Nicotiana tabacum* (Lee *et al.*, *EMBO J.* 7: 1241-1248, 1988)

In order to produce delphinidin or delphinidin-based molecules in rose petals, a number of binary vector constructs were prepared utilising the petunia *F3'5'H* cDNA fragments and various promoter and terminator fragments. The chimaeric petunia *F3'5'H* genes had proved successful in carnation and petunia leading to detectable intact *F3'5'H* transcripts (as detected by Northern blot analysis) and to the production of delphinidin or delphinidin-based molecules pigments. Table 5 summarises the list of binary vector constructs containing petunia *F3'5'H* cDNA fragments.

TABLE 5 Summary of chimaeric petunia *F3'5'H* gene expression cassettes contained in binary vector constructs used in the transformation of roses (see Table 4 for an explanation of abbreviations).

PLASMID	*F3'5'H* GENE	SELECTABLE MARKER GENE
pCGP1452	*AmCHS 5': petHf1: petD8 3'*	*35S 5': SuRB*
pCGP1453	*Mac: petHf1: mas 3'*	*35S 5': SuRB*
pCGP1457	*petD8 5': petHf1: petD8 3'*	*35S 5': SuRB*
pCGP1461	*short petFLS 5': petHf1: petFLS 3'*	*35S 5': SuRB*
pCGP1616	*petRT 5': petHf1: nos 3'*	*35S 5': SuRB*
pCGP1638	*CaMV 35S: petHf1: ocs 3'*	*35S 5': SuRB*
pCGP1623	*mas 35S: petHf1: ocs 3'*	*35S 5': SuRB*
pCGP1860	*RoseCHS 5': petHf1: nos 3'*	*35S 5': SuRB*
pCGP2123	*CaMV 35S: petHf2: ocs 3'*	*35S 5': SuRB*

Isolation of petunia F3'5'H cDNA clones (petHf1 and petHf2)

The isolation and characterisation of cDNA clones of petunia *F3'5'H* (*petHf1* and *petHf2* contained in pCGP602 (Figure 2) and pCGP175 (Figure 3) respectively) (SEQ ID NO:1 and SEQ ID NO:3, respectively) have been described in International Patent Application No. PCT/AU92/00334 and Holton *et al.* (1993a, *supra*).

The plasmids pCGP601 (Figure 2), pCGP602 (Figure 2), pCGP176 (Figure 2) contain homologs of the petunia *petHf1* F3'5'H cDNA clone. The plasmid pCGP601 contains a petunia *F3'5'H petHf1* homolog that includes 52bp of 5' untranslated sequence. The plasmid pCGP602 contains a petunia *F3'5'H petHf1* homolog that includes 125bp of 5' untranslated sequence (SEQ ID NO:1). The plasmid pCGP176 (described in Holton *et al.*, 1993a *supra*) contains a petunia *F3'5'H petHf1* homolog that includes 27bp of 5' untranslated sequence and a further ~127bp of 3' untranslated sequence over the petunia *F3'5'H petHf1* cDNA clone in pCGP602.

Construction of pCGP1303 (petHf1 in pUC19 backbone)

The petunia *F3'5'H* cDNA clone contained in the plasmid pCGP601 (described above) (Figure 2) included 52 bp of 5' untranslated sequence and 141 bp of 3' untranslated sequence including 16 bp of the poly A tail. The plasmid pCGP601 (Figure 2) was firstly linearized by digestion with the restriction endonuclease *Bsp*HI. The ends were repaired and the petunia *F3'5'H petHf1* cDNA clone was released upon digestion with the restriction endonuclease *Fsp*I. The *Bsp*HI recognition sequence encompasses the putative translation initiating codon and the *Fsp*I recognition sequence commences 2 bp downstream from the stop codon. The 1.6 kb fragment containing the petunia *F3'5'H petHf1* cDNA clone was purified and ligated with repaired *Eco*RI ends of pUC19 (New England Biolabs). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1303 (Figure 4).

Construction of pCGP627 (short petHf1 in pBluescript backbone)

The plasmid pCGP176 (Holton *et al.*, 1993a, *supra*) (Figure 2) was digested with the restriction endonuclease *Spe*I and *Eco*RI. The ends were then repaired and allowed to religate. The resulting plasmid was designated as pCGP627 and contained the identical cDNA clone as in pCGP176 except that the restriction endonuclease sites *Pst*I, *Bam*HI and *Sma*I were removed from the multi-cloning site of the pBluescript vector at the 5' end of the cDNA clone.

The binary vector pCGP1452 (AmCHS 5': petHf1: petD8 3')

The plasmid pCGP1452 (Figure 5) contains a chimaeric petunia *F3'5'H* (*petHf1*) gene under the control of a promoter fragment from the *Antirrhinum majus* chalcone synthase gene (*CHS*) (Sommer and Saedler, 1986, *supra*) with a terminator fragment from the petunia phospholipid transfer protein (PLTP) gene (*petD8 3'*) (Holton, 1992, *supra*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* gene of the binary vector, pWTT2132 (DNA Plant Technologies, USA = DNAP) (Figure 6).

Intermediates in the preparation of the binary pCGP1452

<u>The binary vector pWTT2132</u>

The binary vector plasmid pWTT2132 (DNAP) (Figure 6) contains a chimeric gene comprised of a *35S 5'* promoter sequence (Franck *et al.*, 1980, *supra*), ligated with the coding region and terminator sequence for acetolactate synthase (ALS) gene from the *SuRB* locus of tobacco (Lee *et al.*, 1988, *supra*). An ~60bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (Cab 22 gene) (Harpster *et al.*, *MGG*, 212: 182-190, 1988) is included between the *35S 5'* promoter fragment and the *SuRB* sequence.

<u>Construction of pCGP725 (AmCHS 5': petHf1: petD8 3' in pBluescript)</u>

A chimeric petunia *F3'5'H* gene under the control *Antirrhinum majus CHS* (*AmCHS 5'*) promoter with a petunia PLTP terminator (*petD8 3'*) was constructed by cloning the 1.6kb *BclI/FspI* petunia *F3'5'H* (*petHf1*) fragment from pCGP602 (Holton *et al.*, 1993a, *supra*) (Figure 2) between a 1.2 kb *Antirrhinum majus CHS* gene fragment 5' to the site of translation initiation (Sommer and Saedler, 1986, *supra*) and a 0.7 kb *SmaI/XhoI* PLTP fragment (*petD8 3'*) from pCGP13ΔBam (Holton, 1992, *supra*), 3' to the deduced stop codon. The resulting plasmid in a pBluescript II KS (Stratagene, USA) backbone vector was designated pCGP725 (Figure 7).

<u>Construction of pCGP485 and pCGP1452 (AmCHS 5': petHf1: petD8 3' binary vectors)</u>

The chimeric *F3'5'H* gene from pCGP725 (Figure 7) was cloned into the binary vector pCGN1547 containing an *nptII* selectable marker gene cassette (McBride and Summerfelt *Plant Molecular Biology 14:* 269-276, 1990) to create pCGP485. A 3.5 kb fragment containing the *AmCHS 5'*: *petHf1*: *petD8 3'* cassette was released upon digestion of pCGP485 with the restriction endonuclease *PstI*. The overhanging ends were repaired and the purified 3.5 kb fragment was ligated with *SmaI* ends of the binary vector, pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5'*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1452 (Figure 5).

Plant transformation with pCGP1452

The T-DNA contained in the binary vector plasmid pCGP1452 (Figure 5) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1453 (Mac: petHf1: mas 3')

The plasmid pCGP1453 (Figure 8) contains a chimeric petunia *F3'5'H* (*petHf1*) gene under the control of a *Mac* promoter (Comai *et al.*, 1990, *supra*) with a terminator fragment from the mannopine synthase gene of *Agrobacterium* (*mas 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

A 3.9 kb fragment containing the *Mac: petHf1: mas 3'* gene was released from the plasmid pCGP628 (described in International Patent Application No. PCT/AU94/00265) upon digestion with the restriction endonuclease *Pst*I. The overhanging ends were repaired and the purified fragment was ligated with *Sma*I ends of pWTT2132 (DNAP). Correct insertion of the *Mac: petHf1: mas 3'* gene in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1453 (Figure 8).

Plant transformation with pCGP1453

The T-DNA contained in the binary vector plasmid pCGP1453 (Figure 8) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1457 (petD8 5': petHf1: pet D8 3')

The plasmid pCGP1457 (Figure 9) contains a chimaeric petunia *F3'5'H* (*petHf1*) gene under the control of a promoter fragment from the petunia *PLTP* gene (*petD8 5'*) with a terminator fragment from the petunia *PLTP* gene (*petD8 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5'*: *SuRB* gene of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1457

Isolation of petunia D8 genomic clone

Preparation of P. hybrida cv. OGB (Old Glory Blue) genomic library in λ2001

A genomic DNA library was constructed from *Petunia hybrida* cv. OGB DNA in the vector λ2001 (Karn *et al., Gene 32:* 217-224, 1984) using a *Sau*3A partial digestion of the genomic DNA as described in Holton, 1992 *(supra)*. Screening of the OGB genomic library for the petunia D8 gene was as described in Holton, 1992. *supra*.

Isolation of D8 genomic clone OGB2.6

PCR was performed in order to find a non-mutant genomic clone representing D8. Oligo #2 (5' to 3' GTTCTCGAGGAAAGATAATACAAT) (SEQ ID NO:6) and Oligo #4 (5' to 3' CAAGATCGTAGGACTGCATG) (SEQ ID NO:7) were used to amplify D8 gene fragments, across the intron region, using 4 µL of phage suspension from the clones isolated from the primary screening of the OGB genomic library. The reactions were carried out in a total volume of 50 µL containing 1 x Amplification buffer (Cetus), 0.2 mM dNTP mix, <1 µg of template DNA, 50 pmoles of each primer and 0.25 µL of Taq polymerase (5 units/µL - Cetus). The reaction mixtures were overlaid with 30 µL of mineral oil and temperature cycled using a Gene Machine (Innovonics). The reactions were cycled 30 times using the following conditions: 94°C for 1 minute, 55°C for 50 seconds, 72°C for 2 minutes. One quarter of each PCR reaction was run on an agarose gel using TAE running buffer.

Three clones, λOGB-2.4, λOGB-2.5, and λOGB-2.6, gave fragments of approximately 1 kb whereas the mutant clone, λOGB-3.2 (described in Holton, 1992, *supra*), had produced a product of 1.25 kb. The λOGB-2.6 clone was chosen for further analysis.

pCGP382

The genomic clone, λOGB-2.6, contained a single 3.9 kb *Xba*I fragment that hybridized with the D8 cDNA. This *Xba*I fragment was isolated and purified and ligated with the *Xba*I ends of pBluescriptII KS- (Stratagene, USA). Restriction mapping of this clone revealed an internal *Pst*I site 350 bp from the 3' end. However, the "mutant" genomic clone in pCGP13, had an internal *Pst*I near the putative initiating "ATG" of the coding region (approximately 1.5 kb from its 3' end). The difference in the position of the *Pst*I site in both clones suggested that the λOGB-2.6 *Xba*I fragment did not contain the whole genomic sequence of *D8*. A Southern blot was performed on *Pst*I digested λOGB-2.6 DNA, and a fragment of 2.7 kb was found to hybridize with the *D8* cDNA. Restriction endonuclease mapping confirmed that this fragment contained the 3' coding region and flanking sequences.

In order to obtain a fragment containing the whole *D8* genomic sequence, a number of cloning steps were undertaken. The λOGB-2.6 *Pst*I fragment of 2.7 kb was purified and ligated with *Pst*I ends of pBluescriptII KS- (Stratagene, USA). The resultant clone was digested with *Xba*I to remove the 350 bp *Pst*I/*Xba*I fragment. This fragment was replaced by the 3.9 kb *Xba*I fragment from λOGB-2.6 to produce the plasmid pCGP382.

A 3.2 kb fragment containing the promoter region from the *D8 2.6* gene in pCGP382 was released upon digestion with the restriction endonucleases *Hin*dIII and *Nco*I. The fragment was purified and ligated with the 4.8 kb *Nco*I/*Hin*dIII fragment of pJB1 (Bodeau, Molecular and genetic regulation of Bronze-2 and other maize anthocyanin genes. Dissertation, Stanford University, USA, 1994) to produce pCGP1101 containing a *petD85': GUS: nos 3'* cassette.

A 1.6 kb petunia *F3'5'H petHf1* fragment was released from the plasmid pCGP602 (Holton *et al.*, 1993a, *supra*) (SEQ ID NO:1) (Figure 2) upon digestion with the restriction endonucleases *Bsp*HI and *Bam*HI. The fragment was purified and ligated with the 6.2 kb *Nco*I/*Bam*HI fragment of pCGP1101 to produce pCGP1102 containing a *petD8 5': petHf1: nos 3'* expression cassette.

A 0.75 kb *Bam*HI *petD8 3'* fragment (Holton, 1992, *supra*) was purified from the plasmid pCGP13ΔBamHI and ligated with *Bam*HI/*Bgl*II ends of pCGP1102 to produce the plasmid pCGP1107 containing a *petD8 5': petHf1: petD8 3'* expression cassette.

The plasmid pCGP1107 was linearised upon digestion with the restriction endonuclease *Xba*I. The overhanging ends were repaired and then the 5.3 kb fragment containing the *petD8 5': petHf1: petD8 3'* expression cassette was released upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Sma*I/*Pst*I ends of the binary vector pWTT2132 (DNAP) (Figure 6). Correct insertion of the *petD8 5': petHf1: petD8 3' gene* in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1457 (Figure 9).

Plant transformation with pCGP1457

The T-DNA contained in the binary vector plasmid pCGP1457 (Figure 9) was introduced into rose via *Agrobacterium*-mediated transformation.

The binary vector pCGP1461 (short petFLS 5': petHf1: pet FLS 3')

The plasmid pCGP1461 (Figure 10) contains a chimeric petunia *F3'5'H (petHf1)* gene under the control of a promoter fragment from the petunia flavonol synthase *(FLS)* gene (*short petFLS 5'*) with a terminator fragment from the petunia *FLS* gene (*petFLS 3'*). The chimeric petunia *F3'5'H* gene is in a tandem orientation with respect to the *35S 5': SuRB* gene of the binary vector, pWTT2132 (Figure 6).

Intermediates in the preparation of the binary vector pCGP1461

<u>Isolation of petunia FLS gene</u>

Preparation of P. hybrida cv. Th7 genomic library

A *P. hybrida* cv. Th7 genomic library was prepared according to Sambrook *et al.* (1989, *supra*) using a *Sau*3A partial digestion of the genomic DNA. The partially digested DNA was cloned into EMBL-3 lambda vector (Stratagene, USA).

The Th7 genomic DNA library was screened with ^{32}P-labelled fragments of a petunia *FLS* cDNA clone (Holton *et al., Plant J. 4:* 1003-1010, 1993b) using high stringency conditions.

Two genomic clones (*FLS2* and *FLS3*) were chosen for further analysis and found to contain sequences upstream of the putative initiating methionine of the petunia *FLS* coding region with *FLS2* containing a longer promoter region than *FLS3*.

pCGP486

A 6 kb fragment was released upon digestion of the genomic clone *FLS2* with the restriction endonuclease *Xho*I. The fragment containing the short petunia *FLS* gene was purified and ligated with *Xho*I ends of pBluescript SK (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP486.

pCGP487

A 9 kb fragment was released upon digestion of the genomic clone *FLS3* with the restriction endonuclease *Xho*I. The fragment containing the petunia *FLS* gene was purified and ligated with *Xho*I ends of pBluescript SK (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP487.

pCGP717

A 2.2 kb petunia *FLS* promoter fragment upstream from the putative translational initiation site was released from the plasmid pCGP487 upon digestion with the restriction endonucleases *Xho*I and *Pst*I. The fragment generated was purified and ligated with *Xho*I/*Pst*I ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP717.

pCGP716

A 0.95 kb petunia *FLS* terminator fragment downstream from the putative translational stop site was released from the plasmid pCGP487 upon digestion with the restriction endonucleases *Hin*dIII and *Sac*I. The fragment generated was purified and ligated with *Hin*dIII/*Sac*I ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP716.

Construction of pCGP493 (short petFLS 5':petFLS3' expression cassette)

A 1.8 kb fragment containing the short petunia *FLS* promoter fragment was amplified by PCR using the plasmid pCGP717 as template and the T3 primer (Stratagene, USA) and an FLS-*Nco* primer (5' AAA ATC GAT ACC ATG GTC TTT TTT TCT TTG TCT ATA C 3') (SEQ ID NO:19). The PCR product was digested with the restriction endonucleases *Xho*I and *Cla*I and the purified fragment was ligated with *Xho*I/*Cla*I ends of pCGP716. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP493.

Construction of pCGP497 (short petFLS 5': petHf1: petFLS3' expression cassette)

The petunia *F3'5'H* (*petHf1*) cDNA clone was released from the plasmid pCGP627 (described above) upon digestion with the restriction endonucleases *Bsp*HI and *Fsp*I. The *Bsp*HI recognition sequence encompasses the putative translation initiating codon and the *Fsp*I recognition sequence commences 2 bp downstream from the stop codon. The petunia

F3'5'H petHf1 fragment generated was purified and ligated with *Cla*I (repaired ends)/*Nco*I ends of the plasmid pCGP493. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP497.

Construction of pCGP1461 (short petFLS 5': petHf1: petFLS3' binary vector)

The plasmid pCGP497 was linearised upon digestion with the restriction endonuclease *Sac*I. The overhanging ends were repaired and a 4.35 kb fragment containing the *short petFLS 5': petHf1: petFLS3'* gene expression cassette was released upon digestion with the restriction endonuclease *Kpn*I. The fragment generated was purified and ligated with *Pst*I (ends repaired)/*Kpn*I ends of the binary vector pWTT2132 (DNAP) (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1461 (Figure 10).

Plant transformation with pCGP1461

The T-DNA contained in the binary vector plasmid pCGP1461 (Figure 10) was introduced into rose via *Agrobacterium*-mediated transformation.

The binary vector pCGP1616 (petRT 5': petHf1: nos 3')

The plasmid pCGP1616 (Figure 11) contains a chimeric petunia *F3'5'H* (*petHf1*) gene under the control of a promoter fragment from the *P. hybrida 3RT* gene (*petRT 5'*) (Brugliera, 1994, *supra*) with a terminator fragment from the nopaline synthase gene (*nos 3'*) of *Agrobacterium* (Depicker, *et al.*, 1982, *supra*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* gene of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1616

<u>Isolation of petunia 3RT gene</u>

<u>P. hybrida cv. Th7 genomic DNA library construction in EMBL3</u>

A *Petunia hybrida* cv. Th7 genomic library was prepared according to Sambrook *et al.* 1989, *supra* using a *Sau*3A partial digestion of the genomic DNA. The partially digested DNA was cloned into EMBL-3 lambda vector (Stratagene, USA). Screening of the Th7 genomic library for the petunia *3RT* gene was as described in Brugliera, 1994, *supra*.

A 3 kb fragment containing the *petRT 5': petHf1: nos 3'* cassette was released from the plasmid pCGP846 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Pst*I and *Bam*HI. The purified fragment was ligated with *Pst*I/*Bam*HI ends of pWTT2132 (DNAP) (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5'*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1616 (Figure 11).

<u>Plant transformation with pCGP1616</u>

The T-DNA contained in the binary vector plasmid pCGP1616 (Figure 11) was introduced into rose via *Agrobacterium*-mediated transformation.

<u>The binary vector pCGP1623 (mas/35S: petHf1: ocs 3')</u>

The plasmid pCGP1623 (Figure 12) contains a chimeric petunia *F3'5'H* (*petHf1*) gene under the control of the expression cassette contained in pKIWI101 (Janssen and Gardner, 1989, *supra*) consisting of a promoter fragment from the cauliflower mosaic virus *35S* gene (*35S 5'*) with an enhancing sequence from the promoter of the mannopine synthase gene (*mas*) of *Agrobacterium* and a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5'*: *SuRB* gene of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1623

The ~1.6 kb fragment of the petunia *F3'5'H petHf1* cDNA clone contained in the plasmid pCGP1303 (Figure 4) was released upon digestion with the restriction endonucleases *Bsp*HI and *Sma*I. The petunia *F3'5'H petHf1* fragment was purified and ligated with a ~5.9 kb *Nco*I/*Eco*RI (repaired ends) fragment of pKIWI101 (Janssen and Gardner, 1989, *supra*) to produce the plasmid pCGP1619.

A partial digest of the plasmid pCGP1619 with the restriction endonuclease *Xho*I released a 4.9 kb fragment containing the *mas/35S: petHf1: ocs 3'* expression cassette. The fragment was purified and ligated with *Sal*I ends of pWTT2132 (DNAP) (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1623 (Figure 12).

Plant transformation with pCGP1623

The T-DNA contained in the binary vector plasmid pCGP1623 (Figure 12) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1638 (35S 5': petHf1: ocs 3')

The plasmid pCGP1638 (Figure 13) contains a chimeric petunia *F3'5'H* (*petHf1*) gene under the control of a *CaMV 35S* promoter (*35S 5'*) with an octopine synthase terminator (*ocs 3'*). A ~60 bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) (Harpster *et al.*, 1988, *supra*) is included between the *CaMV 35S* promoter fragment and the petunia *F3'5'H petHf1* cDNA clone. The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (Figure 6).

Intermediates in the preparation of the binary vector pCGP1638

Construction of pCGP1273

The plasmid pCGP1273 was constructed by subcloning a ~3kb *Hin*dIII/*Hpa*I fragment containing *35S 5': GUS: ocs 3'* gene from the binary vector pJJ3499 (Jones *et al.*, *Transgenic Research*, *1*: 285-297, 1992) with the *Hin*dIII/*Sma*I ends of the plasmid pBluescript KS II (+) (Stratagene, USA).

Construction of pCGP1634

A ~3kb *Hin*dIII/*Bam*HI fragment containing the *35S 5': GUS: ocs 3'* gene from pCGP1273 was then isolated and ligated with the *Hin*dIII/*Bam*HI ends of the cloning vector pUC19 (New England Biolabs) to create the plasmid pCGP1634.

Construction of pCGP1636

The *GUS* fragment from the plasmid pCGP1634 was removed by digesting pCGP1634 with the restriction endonucleases *Nco*I and *Xba*I and purifying the ~3.7kb fragment containing the *35S 5'* promoter fragment, the *ocs 3'* terminator fragment and the pUC19 vector backbone.

The petunia *F3'5'H petHf1* cDNA clone was released from pCGP1303 (Figure 4) upon digestion with the restriction endonucleases *Bsp*HI and *Xba*I. The resulting ~1.6kb fragment was purified and ligated with the ~3.7kb *Nco*I/*Xba*I fragment from pCGP1634. Correct insertion of the petunia *F3'5'H petHf1* fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid containing a *35S 5': petHf1: ocs 3'* gene was designated pCGP1636.

Construction of pCGP1638

The *35S 5': petHf1: ocs 3'* gene from the plasmid pCGP1636 was released upon digestion of pCGP1636 with the restriction endonucleases *Pst*I and *Eco*RI. The ends were repaired and the ~2.6kb fragment was purified and ligated with the Sam ends of the binary vector, pWTT2132 (DNAP). Correct insertion of the *35S 5': petHf1: ocs 3'* gene in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from

tetracycline-resistant transformants. The plasmid was designated as pCGP1638 (Figure 13).

Plant transformation with pCGP1638

The T-DNA contained in the binary vector plasmid pCGP1638 (Figure 13) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1860 (RoseCHS 5': petHf1: nos 3')

The plasmid pCGP1860 (Figure 14) contains a chimeric petunia *F3'5'H* (*petHf1*) gene under the control of a promoter fragment from the chalcone synthase gene of *Rosa hybrida* (*RoseCHS 5'*) with a terminator fragment from the nopaline synthase gene of *Agrobacterium* (*nos 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1860

Isolation of Rose CHS promoter

A rose genomic DNA library was prepared from genomic DNA isolated from young leaves of *Rosa hybrida* cv. Kardinal.

The Kardinal genomic DNA library was screened with ^{32}P-labelled fragment of rose *CHS* cDNA clone contained in the plasmid pCGP634. The rose *CHS* cDNA clone was isolated by screening of a petal cDNA library prepared from RNA isolated from petals of *Rosa hybrida* cv Kardinal (Tanaka *et al.*, 1995, *supra*) using a petunia *CHS* cDNA fragment as probe (clone *1F11* contained in pCGP701, described in Brugliera *et al.*, 1994, *supra*). Conditions are as described in Tanaka *et al.*, 1995 (*supra*).

A rose genomic clone (*roseCHS20λ*) was chosen for further analysis and found to contain ~6.4 kb of sequence upstream of the putative initiating methionine of the rose *CHS* coding region.

An ~6.4 kb fragment upstream from the translational initiation site was cloned into pBluescript KS (-) (Statagene) and the plasmid was designated as pCGP1114.

The plasmid pCGP1114 was digested with the restriction endonucleases *Hind*III and *Eco*RV to release a 2.7-3.0kb fragment which was purified and ligated with the *Hind*III/*Sma*I ends of pUC19 (New England Biolabs). Correct insertion of the rose *CHS* promoter fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1116. The DNA sequence of the rose *CHS* promoter fragment was determined using pCGP1116 as template (SEQ ID NO:5).

<u>Construction of pCGP197 (RoseCHS 5': GUS : nos 3' in pUC18 backbone)</u>

An ~3.0 kb fragment containing the rose chalcone synthase promoter (*RoseCHS 5'*) was released from the plasmid pCGP1116 upon digestion with the restriction endonucleases *Hin*dIII and *Asp*718. The fragment was purified and ligated with a *Hin*dIII/*Asp*718 fragment from pJB1 (Bodeau, 1994, *supra*) containing the vector backbone, β-glucoronidase (*GUS*) and *nos 3'* fragments. Correct insertion of the rose *CHS* promoter fragment upstream of the *GUS* coding sequence was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP197.

<u>Construction of pCGP200 (RoseCHS 5': petHf1: nos 3' in pUC18 backbone)</u>

A 1.8 kb fragment containing the petunia *F3'5'H* (*petHf1*) fragment was released from the plasmid pCGP1303 (described above) (Figure 4) upon digestion with the restriction endonucleases *Bsp*HI and *Sac*I. The petunia *F3'5'H petHf1* fragment was purified and ligated with *Nco*I/*Sac*I ends of pCGP197. Correct insertion of the petunia *F3'5'H petHf1* fragment between the rose *CHS* promoter and *nos 3'* fragments was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP200.

__Construction of pCGP1860 (RoseCHS 5': petHf1: nos 3' in a binary vector)__

An ~4.9 kb fragment containing the *RoseCHS 5': petHf1: nos 3'* cassette was released from the plasmid pCGP200 upon digestion with the restriction endonuclease *Bgl*II. The fragment was purified and ligated with *Bam*HI ends of the binary vector, pWTT2132 (DNAP) (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1860 (Figure13).

__Plant transformation with pCGP1860__

The T-DNA contained in the binary vector plasmid pCGP1860 (Figure 14) was introduced into rose *via Agrobacterium*-mediated transformation.

__The binary vector pCGP2123 (CaMV 35S: petHf2: ocs 3')__

The plasmid pCGP2123 (Figure 15) contains a chimeric petunia *F3'5'H (petHf2)* gene under the control of a CaMV35S promoter with a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pCGP1988 (Figure 16).

Intermediates in the preparation of the binary vector pCGP2123

__Construction of pCGP1988 (a derivative of the binary vector, pWTT2132)__

The binary vector pCGP1988 (Figure 16) is based on binary vector pWTT2132 (DNAP) (Figure 6) but contains the multi-cloning site from pNEB193 (New England Biolabs). The plasmid pNEB193 was firstly linearized by digestion with the restriction endonuclease *Eco*RI. The overhanging ends were repaired and the multi-cloning fragment was released upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Sal*I (ends repaired)/*Pst*I ends of the binary vector pWTT2132 (DNAP). Correct insertion of the multi-cloning fragment into pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1988 (Figure 16).

__Construction of pCGP2000 (CaMV 35S promoter fragment in pBluescript)__

The plasmid pCGP2000 was an intermediate plasmid containing a cauliflower mosaic virus (CaMV) 35S promoter fragment in a pBluescript SK (Stratagene, USA) backbone. The *CaMV 35S* promoter fragment from pKIWI101 (Janssen and Gardner, 1989, *supra*) was released upon digestion with the restriction endonucleases *Xba*I and *Pst*I. The ~0.35kb fragment generated was purified and ligated with *Xba*I/*Pst*I ends of the vector pBluescript SK. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated as pCGP2000.

__Construction of pCGP2105 (CaMV 35S 5' and ocs 3' fragments in pBluescript)__

The plasmid pCGP2105 (Figure 17) contained a *CaMV 35S* promoter fragment along with a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*) both from pKIWI101 (Janssen and Gardner, 1989, *supra*).

The *ocs* 3' fragment from pKIWI101 (Janssen and Gardner, 1989, *supra*) was isolated by firstly digesting the plasmid pKIWI101 with the restriction endonuclease *Eco*RI, followed by repair of the overhanging ends, and finally by digestion with the restriction endonuclease *Xho*I to release a 1.6 kb fragment. This fragment was then ligated with *Hinc*II/*Xho*I ends of pCGP2000. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated pCGP2105 (Figure 17).

__Construction of pCGP2109 (CaMV 35S: petHf2: ocs 3' gene in pBluescript)__

The plasmid pCGP2109 contained the *CaMV 35S: petHf2: ocs 3'* expression gene cassette in a pBluescript backbone.

The 1.8 kb petunia *F3'5'H petHf2* cDNA clone was released from pCGP175 (Holton *et al.*, 1993a, *supra*) upon digestion with the restriction endonucleases *Xba*I and *Ssp*I. The overhanging ends were repaired and the purified fragment was ligated with *Pst*I (ends

repaired)/*Eco*RV ends of pCGP2105 (described above) (Figure 17). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated pCGP2109.

Construction of pCGP2123 (CaMV 35S: petHf2: ocs 3' binary vector)

The *CaMV 35S: petHf2: ocs 3'* cassette was released from pCGP2109 upon digestion with the restriction endonucleases *Asp*718 and *Xba*I. The overhanging ends were repaired and the resultant ~3.7 kb fragment containing the *CaMV 35S: petHf2: ocs 3' gene* was purified and ligated with repaired ends of *Asp*718 of the binary vector, pCGP1988 (Figure 16). Correct insertion of the *CaMV 35S: petHf2: ocs 3' gene* in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP2123 (Figure 15).

Plant transformation with pCGP2123

The T-DNA contained in the binary vector plasmid pCGP2123 (Figure 15) was introduced into rose *via Agrobacterium*-mediated transformation.

EXAMPLE 5

Analysis of transgenic roses

The transgenic roses produced in the experiments described in Example 4 were grown to flowering. Flowers were collected and the colors of the petals were coded using the Royal Horticultural Society Colour Charts (RHSCC). The anthocyanins were extracted and the anthocyanidins (specifically the presence of delphinidin or delphinidin-based molecules) analysed by TLC and/or HPLC analysis. Total RNA was also isolated from petal tissue and Northern blot analysis was used to detect transcripts of petunia *F3'5'H* transgenes, endogenous rose *CHS* gene and *SuRB* transgene. The results of the transgenic analysis are summarised in Table 6.

Although over 250 transgenic Kardinal roses were produced (Table 6) none produced flowers with a change in color. TLC and/or HPLC analysis failed to detect accumulation of delphinidin or delphinidin-based molecules pigments confirming the absence of efficient F3'5'H activity. Subsequent Northern analysis on total RNA isolated from petal tissue of these transgenic roses revealed either no detectable intact petunia *F3'5'H* (*petHf1* or *petHf2*) transcripts, or in some cases (see footnotes), degraded transcripts. Hybridization of the same membranes with the selectable marker gene (*SuRB*) or with an endogenous rose *CHS* cDNA probe revealed discrete hybridizing transcripts indicating that the total RNA isolated was not degraded. The detection of the *SuRB* transgene transcripts confirmed that the roses were transgenic.

TABLE 6 Results of transgenic analysis of rose petals transformed with the T-DNA from various petunia *F3'5'H* (*petHf1* or *petHf2*) gene expression cassettes.

PLASMID	*F3'5'H* GENE	EVENTS	DEL	RNA
pCGP1452	*AmCHS 5': petHf1: petD8 3'*	34	0/28	0/34[1]
pCGP1453	*Mac: petHf1: mas 3'*	16	0/14	0/13[2]
pCGP1457	*petD8 5': petHf1: petD8 3'*	11	0/11	0/11
pCGP1461	*short petFLS 5': petHf1: petFLS 3'*	11	0/11	0/11
pCGP1616	*petRT 5': petHf1: nos 3'*	4	0/4	0/4
pCGP1623	*mas/35S: petHf1: ocs 3'*	27	0/20	0/12[3]
pCGP1638	*CaMV 35S: petHf1: ocs 3'*	22	0/14	0/14
pCGP1860	*RoseCHS 5': petHf1: nos 3'*	15	0/13	0/13
pCGP2123	*CaMV 35S: petHf2: ocs 3'*	40	0/26	0/10

EVENTS = number of independent transgenic events produced

DEL = number of transgenic events in which delphinidin or delphinidin-based molecules was detected (by TLC or HPLC) in petals over the total number of events analyzed

RNA = number of transgenic events in which intact *F3'5'H* (*petHf1* or *petHf2*) transcripts were detected by Northern blot analysis in total RNA isolated from rose petals over the total number of events analyzed

[1] = Degraded transcripts were detected in 5 of the 34 analyzed

[2] = Degraded transcripts were detected in 8 of the 13 analyzed

[3] = Degraded transcripts were detected in 8 of the 12 analyzed

The fact that no intact petunia *F3'5'H* (*petHf1* or *petHf2*) transcripts were ever detected in transgenic rose petals transformed with the T-DNAs described (Table 6) suggested a number of possibilities:

1. that the RNA isolated was degraded. This was not the case as the RNA had been stained by ethidium bromide and visualised under UV-light. The intact visible ribosomal RNA bands were used as an indicator of the quality of the RNA isolated. Furthermore the detection of full-length transcripts of the endogenous rose *CHS* and *SuRB* transgenes confirmed that the RNA preparation was not degraded.

2. that there was no initiation of transcription of the chimeric *F3'5'H* genes evaluated. This was a possibility with some of the expression cassettes analysed, as no *F3'5'H* transcripts were detected by Northern analysis. However all of the petunia *F3'5'H* expression cassettes had proven to be functional (ie. result in an intact transcript and result in the production of delphinidin-based pigments) in other plants such as carnation and petunia.

3. that the petunia *F3'5'H petHf1* and *petHf2* mRNAs were unstable in roses. This was also a possibility as degraded petunia *F3'5'H* transcripts were detected by Northern analysis in total RNA isolated from petals of some events. However the petunia *petHf1* and *petHf2* mRNAs had been proven to be stable in other plants such as carnation and petunia. Such instability could be due to aberrant translation leading to mRNA turnover, some feature of the sequence inherently unstable in rose cells, some other factor or factors.

There was a need therefore to find suitable promoter fragments that would efficiently drive expression of genes in rose petals and find suitable *F3'5'H* sequences that would result in intact transcripts accumulating in rose petals leading to functional F3'5'H activity and to the production of delphinidin-based pigments.

EXAMPLE 6

Evaluation of promoters in roses

Development of GUS gene expression cassettes.

The evaluation of the promoter and terminator fragments was performed using the GUS reporter gene. Therefore, a number of promoters were linked to the β-glucuronidase reporter gene (*GUS*) (Jefferson *et al.*, 1987, *supra*) and introduced into roses in an attempt to identify expression cassettes that lead to effective initiation of transcription in rose flowers.

A summary of the promoters and terminator fragments evaluated is given in Table 7.

TABLE 7 List of chimaeric *GUS* gene expression cassettes evaluated in roses

PLASMID	*GUS* EXPRESSION CASSETTE	SELECTABLE MARKER GENE	BACKBONE VECTOR
pCGP1307	*petD8 5': GUS: petD8 3'*	*mas 5': nptII : mas 3'*	pCGN1548
pCGP1506	*long petFLS 5': GUS: petFLS 3'*	*nos 5': nptII: nos 3'*	pBIN19
pCGP1626	*chrysCHS 5': GUS: petRT 3'*	*35S 5': SuRB*	pWTT2132
pCGP1641	*petRT 5': GUS: petRT 3'*	*35S 5': SuRB*	pWTT2132
pCGP1861	*RoseCHS 5': GUS: nos 3'*	*35S 5': SuRB*	pWTT2132
pCGP1953	*AmCHS 5': GUS: petD8 3'*	*35S 5': SuRB*	pWTT2132
pWTT2084	*35S 5': GUS: ocs 3'*	*35S 5': SuRB*	pWTT2132

The binary vector pCGP1307 (petD8 5': GUS: petD8 3')

The plasmid pCGP1307 (Figure 18) contains a chimeric *GUS* gene under the control of a promoter and terminator fragment from the petunia *PLTP* gene (*petD8 5'* and *petD8 3'*, respectively). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *mas 5': nptII: mas 3'* selectable marker gene cassette of the binary vector pCGN1548 (McBride and Summerfelt, 1990, *supra*).

Intermediates in the preparation of the binary vector pCGP1307

The *nos 3'* fragment from pCGP1101 (see Example 4) was replaced with the 0.75 kb *petD8 3'* fragment (Holton, 1992, *supra*) to produce the plasmid pCGP1106 containing a *petD8 5': GUS: petD8 3'* expression cassette.

The 5.3 kb fragment containing the *petD8 5': GUS: petD8 3'* expression cassette was released from the plasmid pCGP1106 upon digestion with the restriction endonucleases *Hin*dIII and *Pst*I. The fragment was purified and ligated with *Hin*dIII/*Pst*I ends of the binary vector, pCGN1548 (McBride and Summerfelt, 1990, *supra*). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from gentamycin-resistant transformants. The resulting plasmid was designated as pCGP1307 (Figure 18).

Plant transformation with pCGP1307

The T-DNA contained in the binary vector plasmid pCGP1307 (Figure 18) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1506 (long petFLS 5': GUS: petFLS 3')

The plasmid pCGP1506 (Figure 19) contains a chimeric *GUS* gene under the control of promoter and terminator fragments from the petunia flavonol synthase gene (*petFLS 5'* and *petFLS 3', respectively*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *nos 5': nptII: nos 3'* selectable marker gene cassette of the binary vector pBIN19 (Bevan, *Nucleic Acids Res 12:* 8711-8721, 1984).

Intermediates in the preparation of the binary vector pCGP1506

A 4 kb long petunia *FLS* promoter fragment upstream from the putative translational initiation site was released from the plasmid pCGP486 (described in Example 4) upon digestion with the restriction endonucleases *Xho*I and *Pst*I. The fragment generated was purified and ligated with *Xho*I/*Pst*I ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP715.

Construction of pCGP494 (long petFLS 5':petFLS3' expression cassette)

A 4.0 kb fragment containing the long petunia *FLS* promoter fragment was amplified by PCR using the plasmid pCGP715 as template and the T3 primer (Stratagene, USA) and an FLS-*Nco* primer (5' AAA ATC GAT ACC ATG GTC TTT TTT TCT TTG TCT ATA C 3') (SEQ ID NO:19). The PCR product was digested with the restriction endonucleases *Xho*I and *Cla*I and the purified fragment was ligated with *Xho*I/*Cla*I ends of pCGP716 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP494.

Construction of pCGP496 (long petFLS 5': GUS: petFLS3' expression cassette)

The *GUS* coding sequence from the plasmid pJB1 (Bodeau, 1994, *supra*) was released upon digestion with the restriction endonucleases *Nco*I and *Sma*I. The *GUS* fragment generated was purified and ligated with *Cla*I (repaired ends)/*Nco*I ends of the plasmid pCGP494. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP496.

Construction of pCGP1506 (long petFLS 5': GUS: petFLS3' binary vector)

The plasmid pCGP496 was firstly linearised upon digestion with the restriction endonuclease *Xho*I. The overhanging ends were partially repaired (using only dTTP and dCTP in the reparation reaction) and a 6.7 kb fragment containing the *long petFLS 5':*

GUS: petFLS3' gene expression cassette was released upon digestion with the restriction endonuclease *SacI*. The fragment generated was purified and ligated with *Bam*HI(partially repaired ends using dGTP and dATP in the reparation reaction)/*SacI* ends of the binary vector pBIN19. Correct insertion of the fragment in a tandem orientation with respect to the *nos 5': nptII: nos 3'* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated as pCGP1506 (Figure 19).

Plant transformation with pCGP1506

The T-DNA contained in the binary vector plasmid pCGP1506 (Figure 19) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1626 (chrysCHS 5': GUS: petRT 3')

The plasmid pCGP1626 (Figure 20) contains a chimeric *GUS* gene under the control of promoter fragment from the chalcone synthase gene of chrysanthemum (*chrysCHS 5'*) and a terminator fragment from the 3RT gene of petunia (*petRT 3'*) (Brugliera, 1994, *supra*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1626

Isolation of chrysanthemum CHS promoter

A chrysanthemum genomic DNA library was prepared from genomic DNA isolated from young leaf material of the chrysanthemum *cv* Hero.

The chrysanthemum genomic DNA library was screened with ^{32}P-labelled fragments of a chrysanthemum *CHS* cDNA clone (SEQ ID NO:28) (contained in the plasmid pCGP856) using high stringency conditions. The plasmid pCGP856 contains a 1.5 kb cDNA clone of *CHS* isolated from a petal cDNA library prepared from RNA isolated from the chrysanthemum *cv.* Dark Pink Pom Pom.

A genomic clone (*CHS5*) was chosen for further analysis and found to contain ~3 kb of sequence upstream of the putative initiating methionine of the chrysanthemum *CHS* coding region.

A 4 kb fragment was released upon digestion of the genomic clone *CHS5* with the restriction endonuclease *Hin*dIII. The fragment containing the chrysanthemum *CHS* promoter was purified and ligated with *Hin*dIII ends of pBluescript SK (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1316.

A 2.6 kb chrysanthemum *CHS* promoter fragment upstream from the putative translational initiation site was amplified by PCR using pCGP1316 as template and primers "chrysanCHSATG" (5'-GTTAAGGAAGCCATGGGTGT-3') (SEQ ID NO:8) and the M13 reverse primer (Stratagene, USA). Primer "chrysanCHSATG" incorporated an *Nco*I restriction endonuclease recognition sequence at the putative translation initiation point for ease of cloning. The PCR fragment was purified and ligated with *Eco*RV (dT-tailed) ends of pBluescript KS (Holton and Graham, *Nuc. Acids Res. 19:* 1156, 1990). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1620. The nucleotide sequence of the chrysanthemum *CHS* promoter fragment contained in pCGP1620 is represented as SEQ ID NO:30.

Construction of pCGP1622 (chrysCHS 5': GUS: nos 3' in pUC backbone)

A ~2.5 kb fragment containing the chrysanthemum *CHS* promoter was released from the plasmid pCGP1620 upon digestion with the restriction endonucleases *Nco*I and *Pst*I. The fragment was purified and ligated with a 4.8 kb *NcoI/Pst*I fragment of pJB1 (Bodeau, 1994, *supra*) containing the backbone vector with the *GUS* and *nos 3'* fragments. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1622.

Construction of pCGP1626 (chrysCHS 5': GUS: nos 3' in binary vector)

A ~4.6 kb fragment containing the *chrysCHS 5': GUS: nos 3'* cassette was released from the plasmid pCGP1622 upon digestion with the restriction endonucleases *Pst*I and *Bgl*II. The fragment was purified and ligated with *Pst*I/*Bam*HI ends of the binary vector pWTT2132 (DNAP) (Figure 6). Correct insertion of the cassette in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1626 (Figure 20).

Plant transformation with pCGP1626

The T-DNA contained in the binary vector plasmid pCGP1626 (Figure 20) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1641 (petRT 5': GUS: petRT 3')

The plasmid pCGP1641 (Figure 21) contains a chimeric *GUS* gene under the control of a petunia *3RT* promoter (*petRT 5'*) covering 1.1kb upstream from the putative *3RT* translation initiation codon with a petunia *3RT* terminator (*petRT 3'*) covering 2.5 kb downstream from the 3RT stop codon. The chimeric *GUS* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1641

Isolation of petunia 3RT gene

The isolation of the petunia *3RT* gene corresponding to the *Rt* locus of *P. hybrida* has been described in Brugliera, 1994, *supra*.

Construction of pCGP1625 (CaMV 35S: GUS: petRT 3' cassette)

The intermediate plasmid pCGP1625 contains a *CaMV 35S: GUS: petRT 3'* cassette in a pUC backbone. The 2.5 kb fragment containing a petRT terminator sequences was released from the plasmid pCGP1610 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Bam*HI and *Sac*I. The fragment was purified and ligated with the *Bgl*II/*Sac*I 4.9kb fragment of pJB1 (Bodeau, 1994, *supra*) containing the vector backbone and the *CaMV 35S* promoter and *GUS* fragments. Correct insertion of the petunia *3RT* terminator fragment downstream of the *GUS* fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1625.

Construction of pCGP1628 (petRT 5': GUS: petRT 3' cassette)

A 1.1 kb *petRT* promoter fragment was released from the plasmid pCGP1611 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Nco*I and *Pst*I. The purified fragment was ligated with *Nco*I/*Pst*I ends of the 7kb fragment of pCGP1625 containing the vector backbone and the *GUS* and *petRT 3'* fragments. Correct insertion of the *petRT* promoter fragment upstream of the *GUS* fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1628.

Construction of pCGP1641 (petRT 5': GUS: petRT 3' binary vector)

A 5.4 kb fragment containing the *petRT 5': GUS: petRT 3'* cassette was released from pCGP1628 upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Pst*I ends of the binary vector pWTT2132 (DNAP) (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1641 (Figure 21).

Plant transformation with pCGP1641

The T-DNA contained in the binary vector plasmid pCGP1641 (Figure 21) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1861 (RoseCHS 5': GUS: nos 3')

The plasmid pCGP1861 (Figure 22) contains a chimeric *GUS* gene under the control of a promoter fragment from the *CHS* gene of *R. hybrida* (*RoseCHS 5'*) with a terminator fragment from the *nos* gene of *Agrobacterium* (*nos 3'*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (Figure 6).

An ~5 kb fragment containing the *RoseCHS 5': GUS: nos 3'* cassette was released from pCGP197 (described in Example 4) upon digestion with the restriction endonuclease *Bgl*II. The fragment was purified and ligated with *Bam*HI ends of the binary vector, pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1861 (Figure 22).

Plant transformation with pCGP1861

The T-DNA contained in the binary vector plasmid pCGP1861 (Figure 22) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pCGP1953 (AmCHS 5': GUS: petD8 3')

The plasmid pCGP1953 (Figure 23) contains a chimeric *GUS* gene under the control of a promoter fragment from the *CHS* gene of *Antirrhinum majus* (*AmCHS 5'*) with a petunia *PLTP* terminator (*petD8 3'*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vector pCGP1953

The plasmid pJB1 (Bodeau, 1994, *supra*) was linearised with the restriction endonuclease *Nco*I. The overhanging ends were repaired and the 1.8 kb *GUS* fragment was released upon digestion with *Bam*HI. The *GUS* fragment was purified and was ligated with the 5 kb *Xba*I(ends repaired)/*Bam*HI fragment of pCGP726 containing the pBluescript backbone vector and the *AmCHS 5'* and *petD8 3'* fragments (described in Example 4). Correct insertion of the *GUS* fragment between the *AmCHS 5'* and *petD8 3'* fragments was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated as pCGP1952.

A 3.8 kb fragment containing the *AmCHS 5': GUS: petD8 3'* expression cassette was released from the plasmid pCGP1952 upon digestion with the restriction endonucleases *Eag*I and *Pst*I. The overhanging ends were repaired and the purified fragment was ligated with the repaired ends of an *Asp*718 digested pWTT2132 binary vector (Figure 6). Correct insertion of the fragment in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The plasmid was designated as pCGP1953 (Figure 23).

Plant transformation with pCGP1953

The T-DNA contained in the binary vector plasmid pCGP1953 (Figure 23) was introduced into rose *via Agrobacterium*-mediated transformation.

The binary vector pWTT2084 (35S 5': GUS: ocs 3')

The plasmid pWTT2084 (DNAP) (Figure 24) contains a chimeric *GUS* gene under the control of a *CaMV 35S* promoter (*35S 5'*) with an octopine synthase terminator (*ocs 3'*). An ~60 bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) (Harpster *et al.*, 1988, *supra*) is included between the *CaMV 35S* promoter fragment and the *GUS* clone. The chimeric *GUS* cassette is in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2084.

Plant transformation with pWTT2084

The T-DNA contained in the binary vector plasmid pWTT2084 (Figure 24) was introduced into rose *via Agrobacterium*-mediated transformation.

Transgenic analysis of roses transformed with GUS expression cassettes

Northern blot analysis was performed on total RNA isolated from petals of developmental stages 3 to 4 of transgenic Kardinal roses transformed with the T-DNA of various *GUS* expression cassettes. There was either no accumulating transcript or an intact full-length transcript of the expected size of ~1.8kb as detected by Northern blot hybridisation. The relative levels of *GUS* transcripts accumulating in the rose petals were recorded (see Table 8).

TABLE 8 Summary of Northern analysis on transgenic Kardinal rose flowers (open bud stage) containing *GUS* constructs.

PLASMID	*GUS* REPORTERGENE	SELECTABLE MARKER GENE	*GUS* TRANSCRIPT LEVELS
pCGP1307	*petD8 5': GUS: petD8 3'*	*mas 5': nptII :mas 3'*	–
pCGP1506	*petFLS 5': GUS: petFLS 3'*	*nos 5': nptII: nos 3'*	–
pCGP1626	*chrysCHS 5': GUS: petRT 3'*	*35S 5': SuRB*	++ to +++
pCGP1641	*petRT 5': GUS: petRT 3'*	*35S 5': SuRB*	–
pCGP1861	*RoseCHS 5': GUS: nos 3'*	*35S 5': SuRB*	++++
pCGP1953	*AmCHS 5': GUS: petD8 3'*	*35S 5': SuRB*	–
pWTT2084	*35S 5': GUS: ocs 3'*	*35S 5': SuRB*	+++++

– = no transcripts detected

+ to +++++ = relative levels (low to high) of full-length *GUS* transcript detected by Northern blot analysis

Based on the above results (Table 8), the *CaMV 35S* (*35S 5'*) and rose *CHS* (*RoseCHS 5'*) promoters appear to drive relatively high levels of transcription in rose petals. The chrysanthemum *CHS* promoter (*chrysCHS 5'*) appears to also lead to high transcript levels but not as high as those obtained using *CaMV 35S* or rose *CHS* promoters. Surprisingly, antirrhinum (snapdragon) *CHS* (*AmCHS 5'*), petunia *3RT* (*petRT 5'*), petunia *FLS* (*petFLS 5'*) and petunia *PLTP* –(*petD8 5'*) promoters did not appear to function in rose petals as no *GUS* transcripts were detected with expression cassettes incorporating these promoters. However, these same promoters fused to *petHf1* and/or -*GUS* genes had previously been proven to function well in carnation and petunia leading to relatively high full-length transcript levels and for *petHf1* genes, the production of delphinidin or delphinidin-based molecules pigments. The result obtained with the antirrhinum *CHS* promoter (*AmCHS 5'*) fused with the *GUS* gene was more surprising as promoter regions from homologous genes from two other species (rose and chrysanthemum) appeared to function relatively well in roses. The antirrhinum *CHS* promoter had also been successfully used in conjunction with petunia *F3'5'H* (*petHf1*) to produce the novel violet-colored carnations Florigene Moondust (see International Patent Application No. PCT/AU96/00296).

The evaluation of promoter and terminator fragments fused with the *GUS* gene also provided further evidence to suggest that the petunia *F3'5'H petHf1* and *petHf2* sequences were unstable in roses as constructs containing the petunia *F3'5'H* sequences ligated to the *CaMV 35S*, -rose *CHS* and chrysanthemum *CHS* promoters (which do function in rose) did not result in intact petunia *F3'5'H petHf1* or *petHf2* transcripts in roses (see Table 6).

EXAMPLE 7

Isolation of F3'5'H sequences from species other than petunia

Since the petunia *F3'5'H* sequences had already been proven to function in various plants such as carnation, petunia and tobacco and ultimately resulted in the production of delphinidin-based pigments, it was reasonable to assume that these sequences would also prove functional in roses. There was an assumption that the enzyme activity may vary depending on the background of the species, indeed between cultivars of a given species, that the petunia *F3'5'H* was introduced into. However, there was no expectation that full-

length recombinant petunia *F3'5'H* mRNA would not accumulate. Analysis of the petunia *F3'5'H* nucleotide sequences (*petHf1* and *petHf2*) did not reveal any sequences which might lead to instability and subsequent degradation (Johnson *et al.*, *In A look beyond transcription*, *ASPP*, USA, Bailey-Serres and Gallie, eds, 1998), intron: exon splice junctions (Brendel *et al.*, *In A look beyond transcription*, *ASPP*, USA, Bailey-Serres and Gallie, eds, 1998), or any autocatalytic or degradation trigger sequences reported in the scientific literature to date (*In A look beyond transcription*, *ASPP*, USA, Bailey-Serres and Gallie, eds, 1998). The surprising result suggested that there were factors specific to rose that resulted in petunia *F3'5'H* sequences being unstable.

Since it was not obvious why the petunia *F3'5'H* sequences were unstable in roses but stable in carnation, petunia or tobacco, a number of *F3'5'H* sequences were isolated across a range of families in an attempt to determine whether any *F3'5'H* sequence would be stable in rose and then identify any *F3'5'H* sequences that would lead to the synthesis of stable *F3'5'H* transcripts and F3'5'H activity and ultimately the production of delphinidin-based pigments in roses leading to a change in flower color.

Construction of petal cDNA libraries

Petal cDNA libraries were prepared from RNA isolated from petals from bud to opened flower stages from various species of plants described in Table 9. *Rosa hybrida* is classified in the family Rosaciae, Order Rosales, Subclass Rosidae and so species that produced delphinidin-based pigments and so contained a functional F3'5'H and belonged to the Subclass Rosidae were selected. *Petunia hybrida* is classified in the Family Solanaceae, Order Solanales, Subclass Asteridae and so species from the Subclass Asteridae that produced delphinidin-based pigments were also selected.

TABLE 9 List of flowers from which total RNA was isolated for the preparation of petal cDNA libraries. Information obtained from National Center for Biotechnology Information (NCBI) website under Taxonomy browser (TaxBrowser) as of August 2003.

FLOWER	SPECIES	FAMILY	ORDER	SUBCLASS
gentian	*Gentiana spp.*	Gentianaceae	Gentianales	Asteridae
lavender	*Lavandula spp.*	Lamiaceae	Lamiales	Asteridae
salvia	*Salvia spp.*	Lamiaceae	Lamiales	Asteridae
sollya	*Sollya spp.*	Pittosporaceae	Apiales	Asteridae
kennedia	*Kennedia spp.*	Fabaceae	Fabales	Rosidae
butterfly pea	*Clitoria ternatea*	Fabaceae	Fabales	Rosidae
pansy	*Viola spp.*	Violaceae	Malpighiales	Rosidae

Unless otherwise described, total RNA was isolated from the petal tissue of purple/blue flowers using the method of Turpen and Griffith (*BioTechniques 4:* 11-15, 1986). Poly(A)$^+$ RNA was selected from the total RNA, using oligotex-dT^{TM} (Qiagen) or by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, *Proc. Natl. Acad. Sci. USA 69*: 1408, 1972).

In general a λZAPII/ Gigapack II Cloning kit (Stratagene, USA) (Short *et al., Nucl. Acids Res. 16:* 7583-7600, 1988) was used to construct directional petal cDNA libraries in λZAPII using around 5 μg of poly(A)$^+$ RNA isolated from petal as template. The total number of recombinants obtained was generally in the order of 1 x 10^5 to 1 x 10^6.

After transfecting XL1-Blue MRF' cells, the packaged cDNA mixtures were plated at around 50,000 pfu per 15 cm diameter plate. The plates were incubated at 37°C for 8 hours, and the phage were eluted in 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 8.0, 0.01% (w/v) gelatin (Phage Storage Buffer (PSB)) (Sambrook *et al.*, 1989, *supra*). Chloroform was added and the phages stored at 4°C as amplified libraries.

In general around 100,000 pfu of the amplified libraries were plated onto NZY plates (Sambrook *et al.*, 1989, *supra*) at a density of around 10,000 pfu per 15 cm plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After incubation at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Plasmid Isolation

Helper phage R408 (Stratagene, USA) was used to excise pBluescript phagemids containing cDNA inserts from amplified λZAPII or λZAP cDNA libraries using methods described by the manufacturer.

Screening of petal cDNA Libraries

Prior to hybridization, duplicate plaque lifts were washed in prewashing solution (50 mM Tris-HCl pH7.5, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) sarcosine) at 65°C for 30 minutes; followed by washing in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% (w/v) SDS.

The membrane lifts from the petal cDNA libraries were hybridized with ^{32}P-labelled fragments of a 1.6 kb *Bsp*HI/*Fsp*I fragment from pCGP602 (Figure 2) (SEQ ID NO: 1) containing the petunia *F3'5'H petHf1* cDNA clone (Holton *et al.*, 1993a, *supra*).

Hybridization conditions included a prehybridization step in 10% v/v formamide, 1 M NaCl, 10% w/v dextran sulphate, 1% w/v SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragments (each at 1×10^6 cpm/mL) were then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% w/v SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Strongly hybridizing plaques were picked into PSB (Sambrook *et al.*, 1989, *supra*) and rescreened to isolate purified plaques, using the plating and hybridization conditions as described for the initial screening of the cDNA library. The plasmids contained in the λZAPII or λZAP bacteriophage vectors were rescued and sequence data was generated from the 3' and 5' ends of the cDNA inserts. New *F3'5'H* cDNA clones were identified based on sequence similarity to the petunia *F3'5'H petHf1* cDNA clone.

The cDNA clones isolated were given plasmid designation numbers as described in Table 10.

TABLE 10 Plasmid numbers and SEQ ID NO. of *F3'5'H* cDNA clones isolated from various species

SPECIES	CLONE	PLASMID NUMBER	FIGURE NUMBER	SEQ ID NO.
Viola spp.	BP#18	pCGP1959	25	9
Viola spp.	BP#40	pCGP1961	26	11
Salvia spp.	Sal#2	pCGP1995	31	13
Salvia spp.	Sal#47	pCGP1999	32	15
Sollya spp.	Soll#5	pCGP2110	37	17
Kennedia spp.	Kenn#31	pCGP2231	40	26
Clitoria ternatea	BpeaHF2	pBHF2F4	43	20
Gentiana triflora	Gen#48	pG48	47	22
Lavandula nil	LBG	pLHF8	51	31

Viola (pansy) F3'5'H constructs

Isolation of F3'5'H cDNA clones from petals of Viola spp. (pansy)

Total RNA and poly $(A)^+$ RNA was isolated from petals of young buds of *Viola spp.* cultivar black pansy as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA) and screened as described above. Two full-length pansy *F3'5'H* cDNA clones (*BP#18* (SEQ ID NO:9) in pCGP1959 (Figure 25)

and BP#40 (SEQ ID NO:11) in pCGP1961 (Figure 26)) were identified by sequence similarity to the petunia *F3'5'H petHf1* cDNA clone (SEQ ID NO:1). The BP#18 and BP#40 shared 82% identity at the nucleotide level. Comparison of the nucleotide sequence of pansy *F3'5'H* clones (BP#18 and BP#40) with that of the petunia *F3'5'H* revealed around 60% identity to the petunia *F3'5'H petHf1* clone and 62% identity to the petunia *F3'5'H petHf2* clone.

***<u>The binary vectors, pCGP1972 and pCGP1973</u>* (*AmCHS 5'*: BP#18 or BP#40: *petD8 3'*)**

The plasmids pCGP1972 (Figure 27) and pCGP1973 (Figure 28) contain the pansy *F3'5'H* cDNA clone (BP#18 and BP#40, respectively) between an *A. majus* (snapdragon) *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*). The chimeric *F3'5'H* genes are in tandem with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonuclease *Bam*HI. The ends were repaired and the linearised plasmid was further digested with the restriction endonuclease *Xba*I. The ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* fragments was purified and ligated with the ~1.6kb *Kpn*I (ends repaired)/*Xba*I fragment containing the pansy *F3'5'H* BP#18 or BP#40 cDNA clone from pCGP1959 or pCGP1961, respectively to produce pCGP1970 and pCGP1971, respectively. The *AmCHS 5': pansy F3'5'H: petD8 3'* cassette was then isolated from pCGP1970 or pCGP1971 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* genes were released upon digestion with the restriction endonuclease *Eco*RV. The purified fragments were then ligated with *Asp*718 (repaired ends) of the binary vector pWTT2132 (DNAP). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP1972 (Figure 27) and pCGP1973 (Figure 28), respectively.

Carnation and petunia transformation with pCGP1972 and 1973

The T-DNAs contained in the binary vector plasmids pCGP1972 (Figure 27) and pCGP1973 (Figure 28) were introduced separately into *Dianthus caryophyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 via *Agrobacterium*-mediated transformation.

The binary vectors, pCGP1967 and pCGP1969 (CaMV 35S: pansy F3'5'H: ocs 3')

The binary vectors pCGP1967 (Figure 29) and pCGP1969 (Figure 30) contain chimeric *CaMV 35S: pansy F3'5'H: ocs 3'* genes in tandem with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector, pWTT2132 (DNAP) (Figure 6).

Intermediates in the preparation of the binary vectors pCGP1967 and pCGP1969

The plasmids pCGP1959 (Figure 25) and pCGP1961 (Figure 26) were firstly linearized upon digestion with the restriction endonuclease *Kpn*I. The overhanging *Kpn*I ends were repaired and the pansy *F3'5'H* cDNA clones, BP#18 and BP#40, were released upon digestion with the restriction endonuclease *Pst*I. The ~1.6 kb fragments generated were ligated with an ~5.9 kb *Eco*RI (repaired ends)/*Pst*I fragment of pKIWI101 (Janssen and Gardner, 1989, *supra*). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmids were designated pCGP1965 and pCGP1966, respectively.

The plasmids pCGP1965 and pCGP1966 were firstly partially digested with the restriction endonuclease *Xho*I. The resulting fragments were further digested with the restriction endonuclease *Xba*I. The overhanging ends were repaired and the 3.6kb fragments containing the *CaMV 35S: pansy F3'5'H: ocs 3'* chimeric genes were isolated and ligated with *Asp*718 repaired ends of pWTT2132 (Figure 6). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP1967 (Figure 29) and pCGP1969 (Figure 30), respectively.

Rose transformation with pCGP1967 and pCGP1969

The T-DNAs contained in the binary vector plasmids pCGP1967 (Figure 29) and pCGP1969 (Figure 31) were introduced separately into *Rosa hybrida cv.* Kardinal and Soft Promise via *Agrobacterium*-mediated transformation. The T-DNA contained in the binary vector plasmids pCGP1969 (Figure 31) was also introduced into *Rosa hybrida cv.* Pamela and Medeo via *Agrobacterium*-mediated transformation.

Salvia F3'5'H constructs

Isolation of a F3'5'H cDNA clone from petals of Salvia spp.

Total RNA and poly $(A)^+$ RNA was isolated from young petal buds of *Salvia spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). Two full-length salvia *F3'5'H* cDNA clones (Sal#2 (SEQ ID NO:13) in pCGP1995 (Figure 31) and Sal#47 (SEQ ID NO:15) in pCGP1999 (Figure 32)) were identified by sequence similarity with the petunia *F3'5'H petHf1* cDNA clone. The Sal#2 and Sal#47 shared 95% identity at the nucleotide level. Comparison of the nucleotide sequence of salvia *F3'5'H* clones (Sal#2 and Sal#47) with that of the petunia *F3'5'H* revealed around 57% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 58% identity to the petunia *F3'5'H petHf2* clone (SEQ ID NO:3).

The binary vectors, pCGP2121 and pCGP2122

(AmCHS 5': Salvia F3'5'H #2 or #47: petD8 3')

The plasmids pCGP2121 (Figure 33) and pCGP2122 (Figure 34) contain the salvia *F3'5'H* cDNA clones (Sal#2 and Sal#47, respectively) between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pWTT2132 (DNAP) (Figure 6).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonuclease *Bam*HI. The ends were repaired and the linearised plasmid was further digested with the restriction endonuclease *Xba*I. The ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* fragments was purified and ligated with the ~1.6kb *Xho*I/*Bam*HI (ends repaired) fragment from pCGP1995 (Figure 31) containing the salvia *F3'5'H* #2 or *Xho*I/*Eco*RI (ends repaired) fragment from pCGP1999 (Figure 32) containing the salvia *F3'5'H* #47, respectively to produce pCGP2116 and pCGP2117, respectively.

The *AmCHS 5'*: *salvia F3'5'H*: *petD8 3'* cassette was isolated from pCGP2116 or pCGP2117 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearized plasmid were repaired and then the chimeric *F3'5'H* gene cassettes were released upon digestion with the restriction endonuclease *Eco*RV. The ~3.6kb purified fragments were then ligated with *Asp*718 repaired ends of the binary vector pCGP1988 (Figure 16) (described in Example 4). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP2121 (Figure 33) and pCGP2122 (Figure 34), respectively.

Carnation and petunia transformation with pCGP2121 and pCGP2122

The T-DNAs contained in the binary vector plasmids pCGP2121 (Figure 33) and pCGP2122 (Figure 34) were introduced separately into *Dianthus caryophyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

The binary vectors, pCGP2120 and pCGP2119 (CaMV 35S: salvia F3'5'H: ocs 3')

The binary vectors pCGP2120 (Figure 35) and pCGP2119 (Figure 36) contain chimeric *CaMV 35S: salvia F3'5'H: ocs 3'* gene cassettes in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

Intermediates in the preparation of the binary vectors pCGP2120 and pCGP2119

The plasmids pCGP1995 (Figure 31) and pCGP1999 (Figure 32) were firstly linearized upon digestion with the restriction endonuclease *Xho*I. The overhanging *Xho*I ends were repaired and then the salvia *F3'5'H* cDNA clones Sal#2 or Sal#47 were released upon digestion with the restriction endonuclease *Eco*RI. In the case of pCGP1995 a partial digest with *Eco*RI was undertaken. The ~1.7 kb fragments were ligated with the *Cla*I (repaired ends)/*Eco*RI ends of pCGP2105 (Figure 17). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmids were designated pCGP2112 and pCGP2111, respectively.

The plasmids pCGP2112 and pCGP2111 were digested with the restriction endonucleases *Xho*I and *Xba*I. The resulting overhanging ends were repaired and ~3.6 kb fragments containing the *CaMV 35S: salvia F3'5'H: ocs 3'* chimeric genes were isolated and ligated with *Asp*718 repaired ends of the binary vector, pCGP1988 (described in Example 4). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP2120 (Figure 35) and pCGP2119 (Figure 36), respectively.

Rose transformation with pCGP2120 and pCGP2119

The T-DNAs contained in the binary vector plasmids pCGP2120 (Figure 35) and pCGP2119 (Figure 36) were introduced separately into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Sollya F3'5'H constructs

Isolation of a F3'5'H cDNA clone from petals of Sollya spp.

Total RNA and poly (A)$^+$ RNA was isolated from young petal buds of *Sollya spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). One full-length Sollya *F3'5'H* cDNA clone (Soll#5 (SEQ ID NO:17) in pCGP2110 (Figure 37)) was identified by sequence similarity to the petunia *F3'5'H petHf1* cDNA clone. Comparison of the nucleotide sequence of the

sollya *F3'5'H* clone with that of the petunia *F3'5'H* revealed around 48% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 52% identity to the petunia *F3'5'H petHf2* clone (SEQ ID NO:3).

<u>The binary vector pCGP2130 (AmCHS 5': Sollya F3'5'H: petD8 3')</u>

The plasmid pCGP2130 (Figure 38) contains the sollya *F3'5'H* SolI#5 cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in a tandem orientation with respect to the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonucleases *Xba*I *and* *Bam*HI. The ends were repaired the ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* fragments was purified and ligated with the repaired ends of the ~1.6kb *Asp*718/*Pst*I fragment from pCGP2110 containing the sollya *F3'5'H* cDNA clone to produce pCGP2128. Correct insertion of the sollya F3'5'H fragment in tandem with the *AmCHS 5'* and *petD8 3'* fragments was confirmed by restriction endonuclease mapping.

The *AmCHS 5'*: sollya *F3'5'H: petD8 3'* gene cassette was then isolated from pCGP2128 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearized plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The ~3.5kb purified fragment was then ligated with *Asp*718 (repaired ends) of the binary vector pCGP1988 (described in Example 4) (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2130 (Figure 38).

<u>Carnation and petunia transformation with pCGP2130</u>

The T-DNA contained in the binary vector plasmid pCGP2130 (Figure 38) was introduced into *Dianthus caryophyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

The binary vector pCGP2131 (CaMV 35S: sollya F3'5'H: ocs 3')

The binary vector pCGP2131 (Figure 39) contains a chimeric *CaMV 35S: sollya F3'5'H: ocs 3'* gene in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

Intermediates in the preparation of the binary vector pCGP2131

The plasmid pCGP2110 was firstly linearized upon digestion with the restriction endonuclease *Asp*718. The overhanging ends were repaired and then the sollya *F3'5'H* cDNA clone was released upon digestion with the restriction endonuclease *Pst*I. The ~1.7 kb fragment was ligated with the *Eco*RV/*Pst*I ends of pCGP2105 (Figure 17). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2129.

A 3.6 kb fragment containing the *CaMV 35S: sollya F3'5'H: ocs 3'* chimeric gene was released upon digestion with the restriction endonucleases *Asp*718 and *Xba*I The overhanging ends were repaired and the purified fragment was ligated with of *Asp*718 repaired ends of the binary vector, pCGP1988 (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2131 (Figure 39).

Rose transformation with pCGP2131

The T-DNA contained in the binary vector plasmid pCGP2131 (Figure 39) was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Kennedia F3'5'H constructs

Isolation of a F3'5'H cDNA clone from petals of Kennedia spp.

Total RNA and poly (A)$^+$ RNA was isolated from young petal buds of *Kennedia spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). One full-length kennedia *F3'5'H* cDNA clone (*Kenn#31* in pCGP2231 (Figure 40)) (SEQ ID NO:26) was identified by sequence similarity to the petunia *F3'5'H petHf1* cDNA clone. Comparison of the nucleotide sequence of the kennedia *F3'5'H* clone with that of the petunia *F3'5'H* revealed around 64% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 60% identity to the petunia *F3'5'H petHf2* clone (SEQ ID NO:3).

The binary vector pCGP2256 (AmCHS 5': kennedia F3'5'H: petD8 3')

The plasmid pCGP2256 (Figure 41) contains the kennedia *F3'5'H* (*Kenn#31*) cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonucleases *Xba*I and *Bam*HI. The ends were repaired the ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* fragments was purified and ligated with the repaired ends of the ~1.8kb *XhoI/Bam*HI fragment from pCGP2231 containing the kennedia *F3'5'H* cDNA clone to produce pCGP2242. Correct insertion of the kennedia *F3'5'H* fragment in tandem with the *AmCHS 5'* and *petD8 3'* fragments was confirmed by restriction endonuclease mapping.

The *AmCHS 5': kennedia F3'5'H: petD8 3'* cassette was then isolated from pCGP2242 by digesting with the restriction endonucleases *Not*I and *Eco*RI. The ends were repaired and the ~3.7kb purified fragment was then ligated with *Asp*718 repaired ends of the binary vector pCGP1988 (described in Example 4) (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from

tetracycline-resistant transformants. The resulting plasmid was designated pCGP2256 (Figure 41).

Petunia transformation with pCGP2256

The T-DNA contained in the binary vector plasmid pCGP2256 (Figure 41) was introduced into *Petunia hybrida* cv. Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

The binary vector pCGP2252 (CaMV 35S: kennedia F3'5'H: ocs 3')

The binary vector pCGP2252 (Figure 42) contains a chimeric *CaMV 35S: kennedia F3'5'H: ocs 3'* gene in tandem with the *35S 5': SuRB* selectable marker cassette of the binary vector pCGP1988 (Figure 16).

Intermediates in the preparation of the binary vector pCGP2252

The plasmid pCGP2231 was firstly linearized upon digestion with the restriction endonuclease *Xho*I. The overhanging ends were repaired and then the kennedia *F3'5'H* cDNA clone was released upon digestion with the restriction endonuclease *Pst*I. The ~1.7 kb fragment was ligated with the *Cla*I (repaired ends)/*Pst*I ends of pCGP2105 (Figure 17). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2236.

A 3.6 kb fragment containing the *CaMV 35S: kennedia F3'5'H: ocs 3'* chimeric gene cassette was released from the plasmid pCGP2236 upon digestion with the restriction endonucleases *Xho*I and *Not*I. The overhanging ends were repaired and the purified fragment was ligated with *Asp*718 repaired ends of the binary vector, pCGP1988 (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2252 (Figure 42).

Rose transformation with pCGP2252

The T-DNA contained in the binary vector plasmid pCGP2252 (Figure 42) was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Butterfly pea F3'5'H constructs

<u>Isolation of a F3'5'H cDNA clone from petals of Clitoria ternatea (butterfly pea)</u>

Construction of butterfly pea petal cDNA library

A blue variety of *Clitoria ternatea* (butterfly pea, the seeds were kindly provided by Osaka Botanical Garden) was grown in a field in Osaka. Total RNA was isolated from fresh and pigmented petals at a pre-anthesis stage as described above. PolyA$^+$ RNA was prepared using Oligotex (Takara) according to the manufacturer's recommendation. A petal cDNA library of butterfly pea was constructed from the polyA$^+$ RNA using a directional λZAP-cDNA synthesis kit (Stratagene, USA) following the manufacturer's protocols.

<u>Screening of butterfly pea cDNA library for a F3'5'H cDNA clone</u>

The butterfly pea petal cDNA library was screened with DIG-labelled petunia *F3'5'H petHf1* cDNA clone as described previously (Tanaka *et al., Plant Cell Physiol. 37:* 711-716, 1996). Two cDNA clones that showed high sequence similarity to the petunia *F3'5'H petHf1* were identified. The plasmid containing the longest cDNA clone was designated pBHF2 and the cDNA clone was sequenced. Alignment between the deduced amino acid sequences of the butterfly pea *F3'5'H* clone and the petunia *F3'5'H petHf1* clone (SEQ ID NO:2) revealed that the butterfly pea *F3'5'H* cDNA (contained in pBHF2) did not represent a full-length cDNA and lacked first 2 bases of the putative initiation codon. These two bases along with a *Bam*HI restriction endonuclease recognition site were added to the cDNA clone using PCR and a synthetic primer, 5'-GGGATCCAACAATGTTCCTTCTAAGAGAAAT-3' [SEQ ID NO:25] as described previously (Yonekura-Sakakibara *et al., Plant Cell Physiol. 41:* 495-502, 2000). The resultant fragment was digested with the restriction endonucleases *Bam*HI and *Pst*I and the subsequent DNA fragment of about 200 bp was recovered. The DNA fragment was ligated with a 3.3 kb fragment of *Bam*HI/*Eco*RI digested pBHF2 to yield pBHF2F (Figure 43). The DNA sequence was confirmed to exclude errors made during PCR (SEQ ID NO:20).

Comparison of the nucleotide sequence of butterfly pea *F3'5'H* clone (SEQ ID NO:20) with that of the petunia *F3'5'H* revealed around 59% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 62% identity to the petunia *F3'5'H petHf2* clone (SEQ ID NO:3).

The binary vector pCGP2135 (AmCHS 5': butterfly pea F3'5'H: petD8 3')

The plasmid pCGP2135 (Figure 44) contains the butterfly pea *F3'5'H* cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonucleases *Xba*I and *Bam*HI. The ends were repaired the ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* fragments was purified and ligated with the repaired ends of the ~1.6kb *Xho*I/*Bam*HI fragment from pBHF2F (Figure 43) containing the *butterfly pea F3'5'H* cDNA clone to produce pCGP2133. Correct insertion of the *butterfly pea F3'5'H* fragment in tandem with the *AmCHS 5'* and *petD8 3'* fragments was confirmed by restriction endonuclease mapping.

The *AmCHS 5': butterfly pea F3'5'H: petD8 3'* cassette was then isolated from pCGP2133 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The ~3.6kb purified fragment was then ligated with *Asp*718 repaired ends of the binary vector pCGP1988 (described in Example 4) (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2135 (Figure 44).

Carnation and petunia transformation with pCGP2135

The T-DNA contained in the binary vector plasmid pCGP2135 (Figure 44) was introduced into *Dianthus caryophyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida* cv. Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

The binary vector pBEBF5 (eCaMV 35S: Butterfly pea F3'5'H: nos 3')

The binary vector, pBE2113-GUS contains a *GUS* coding region between an enhanced *CaMV 35S* promoter and *nos* terminator in a pBI121 binary vector (Mitsuhara *et al.*, 1996, *supra*). The plasmid pBE2113-GUS was digested with the restriction endonuclease *Sac*I. The overhanging ends were repaired and then ligated with a *Sal*I linker to yield pBE2113-GUSs. The 1.8 kb *Bam*HI-*Xho*I fragment from pBHF2F was ligated with *Bam*HI-*Sal*I digested pBE2113-GUSs to create pBEBF5 (Figure 45).

Rose transformation with pBEBF5

The T-DNA contained in the binary vector plasmid pBEBF5 (Figure 45) was introduced into *Rosa hybrida* cultivar Lavande *via Agrobacterium*-mediated transformation.

The binary vector pCGP2134 (CaMV 35S: butterfly pea F3'5'H: ocs 3')

The binary vector pCGP2134 (Figure 46) contains a chimeric *CaMV 35S: butterfly pea F3'5'H: ocs 3'* gene cassette in a tandem orientation with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pCGP1988 (Figure 16).

Intermediates in the preparation of the binary vector pCGP2134

The butterfly pea *F3'5'H* cDNA clone was released upon digestion of the plasmid pBHF2F (Figure 43) with the restriction endonucleases *Xho*I and *Bam*HI. The overhanging ends were repaired and the ~1.7 kb fragment was ligated with the *Pst*I (repaired ends)/*Eco*RV ends of pCGP2105 (described in Example 4) (Figure 17). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2132.

An ~3.6 kb fragment containing the *CaMV 35S: butterfly pea F3'5'H: ocs 3'* chimeric gene cassette was released upon digestion with the restriction endonucleases *Xho*I and *Xba*I. The overhanging ends were repaired and the purified fragment was ligated with *Asp*718 repaired ends of the binary vector, pCGP1988 (described in Example 4) (Figure 16). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2134 (Figure 46).

Rose transformation with pCGP2134

The T-DNA contained in the binary vector plasmid pCGP2134 (Figure 46) was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Gentia F3'5'H constructs

Isolation of a F3'5'H cDNA clone from petals of Gentiana triflora (gentian).

Construction and screening of a gentian petal cDNA library

The isolation of a gentian cDNA encoding *F3'5'H* has been described previously (Tanaka *et al.*, 1996, *supra*) and is contained within the plasmid pG48 (Figure 47). Comparison of the nucleotide sequence of the gentia F3'5'H clone (*Gen#48*) (SEQ ID NO:22) contained in the plasmid pG48 (Figure 47) with that of the petunia *F3'5'H* revealed around 61% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 64% identity to the petunia *F3'5'H petHf2* clone (SEQ ID NO:3).

The binary vector pCGP1498 (AmCHS 5': gentia F3'5'H: petD8 3')

The plasmid pCGP1498 (Figure 48) contains the gentia F3'5'H (*Gen#48*) cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pWTT2132 (Figure 6).

The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (described in Example 4) (Figure 7) was removed by initially digesting pCGP725 with the restriction endonucleases *Xba*I and *Bam*HI. The ends were repaired the ~4.9kb fragment containing the vector with the

AmCHS 5' and *petD8 3'* fragments was purified and ligated with the repaired ends of the ~1.7 kb *XhoI/Bam*HI fragment from pG48 (Figure 47) containing the *gentia F3'5'H* cDNA clone to produce pCGP1496. Correct insertion of the *gentia F3'5'H* fragment in tandem with the *AmCHS 5'* and *petD8 3'* fragments was confirmed by restriction endonuclease mapping.

The *AmCHS 5'*: gentia F3'5'H: *petD8 3'* cassette was then isolated from pCGP1496 by firstly digesting with the restriction endonuclease *Not*I. The overhanging ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The ~3.6kb purified fragment was then ligated with *Asp*718 repaired ends of the binary vector pWTT2132 (Figure 6). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP1498 (Figure 48).

Carnation and petunia transformation with pCGP1498

The T-DNA contained in the binary vector plasmid pCGP1498 (Figure 48) was introduced into *Dianthus caryophyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida* cv. Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

The binary vector pBEGHF48 (eCaMV 35S: gentia F3'5'H: nos 3')

The gentia F3'5'H cDNA clone was released by digestion of the plasmid pG48 with the restriction endonucleases *Bam*HI and *Xho*I. The resulting ~1.7 kb DNA fragment was isolated and ligated with *Bam*HI/*Sal*I digested pBE2113-GUSs (Mitsuhara *et al*., 1996, *supra*) to create pBEGHF48 (Figure 49).

Rose transformation with pBEGHF48

The T-DNA contained in the binary vector plasmid pBEGHF48 (Figure 49) was introduced into *Rosa hybrida cv.* Lavande *via Agrobacterium*-mediated transformation.

<u>The binary vector pCGP1982 (CaMV 35S: gentia F3'5'H: ocs 3')</u>

The binary vector pCGP1982 (Figure 50) contains a chimeric *CaMV 35S: gentia F3'5'H: ocs 3'* gene cassette in tandem with the *35S 5': SuRB* selectable marker gene cassette of the binary vector pWTT2132 (Figure 6).

Intermediates in the preparation of the binary vector pCGP1982

The plasmid pG48 (Figure 47) was linearised upon digestion with the restriction endonuclease *Asp*718. The overhanging ends were repaired and then the gentia F3'5'H cDNA clone (*Gen#48*) was released upon digestion with the restriction endonuclease *Bam*HI. The ~1.7 kb fragment was ligated with the 5.95kb *Eco*RI (repaired ends)/*Bam*HI fragment of pKIWI101 (Janssen and Gardner, 1989, *supra*). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP1981.

An ~3.6 kb fragment containing the *CaMV 35S: gentia F3'5'H: ocs 3'* chimeric gene cassette was released upon digestion of the plasmid pCGP1981 with the restriction endonucleases *Xho*I and *Xba*I The overhanging ends were repaired and the purified fragment was ligated with repaired ends of *Asp*718 digested binary vector, pWTT2132. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP1982 (Figure 50).

<u>Rosa transformation with pCGP1982</u>

The T-DNA contained in the binary vector plasmid pCGP1982 (Figure 50) was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Lavender F3'5'H constructs

Isolation of a F3'5'H cDNA clone from petals of Lavandula nil (lavender)

Construction of lavender petal cDNA library

Cut flowers of a violet variety of *Lavandula nil* were purchased from a florist. Total RNA was isolated from fresh and pigmented petals as described above. PolyA+ RNA was prepared using Oligotex (Takara) according to the manufacturer's recommendations. A petal cDNA library of lavender was constructed from the polyA$^+$ RNA using a directional λZAP-cDNA synthesis kit (Stratagene, USA) following the manufacturer's protocols.

Screening of lavender cDNA library for a F3'5'H cDNA clone

The lavender petal cDNA library was screened with DIG labelled petunia *F3'5'H petHf1* cDNA clone as described previously (Tanaka *et al.*1996, *supra*). One cDNA clone (*LBG*) that showed high similarity to petunia *F3'5'H petHf1* was identified and the plasmid was designated pLHF8 (Figure 51). The nucleotide sequence of the lavender F3'5'H (*LBG*) cDNA clone was designated as SEQ ID NO: 31.

Comparison of the nucleotide sequence of lavender *F3'5'H* clone with that of the petunia *F3'5'H* cDNA clones revealed around 59% identity to the petunia *F3'5'H petHf1* clone (SEQ ID NO:1) and 60% identity to the petunia *petHf2* clone (SEQ ID NO:3).

The binary vector pBELF8 (eCaMV 35S: lavender F3'5'H: nos 3')

The plasmid of pLHF8 (Figure 51) was digested with the restriction endonucleases *Bam*HI and *Xho*I to release a DNA fragment of approximately 1.8 kb. The ~1.8kb purified fragment from pLHF8 was then ligated with the *Bam*HI-*Sal*I digested ends of the plasmid pBE2113-GUSs (described above) to create pBELF8 (Figure 52).

Rose transformation with pBELF8

The T-DNA contained in the binary vector plasmid pBELF8 (Figure 52) was introduced into *Rosa hybrida* cultivar Lavande *via Agrobacterium*-mediated transformation.

EXAMPLE 8

Analysis of transgenic carnation, petunia and rose

The transgenic plants produced in the experiments described in Example 7 were grown to flowering. Flowers were collected and the colors of the petals were coded using the Royal Horticultural Society Colour Charts (RHSCC). The anthocyanins were extracted and the anthocyanidins analysed by spectrophotometric, TLC and/or HPLC analysis. Total RNA was also isolated from petal tissue of the appropriate stages of flower development and Northern blot analysis was used to detect transcripts of *F3'5'H* transgenes. The results of the transgenic analysis are summarised in Tables 11, 12 and 13.

Carnation

The *F3'5'H* genes described in Example 7 were evaluated for their ability to lead to the production of delphinidin-based pigments in carnation petals. Two carnation cultivars, Kortina Chanel (KC) and Monte Lisa (ML), were used in the transformation experiments. The carnation cultivar Kortina Chanel produces pink colored flowers that normally accumulate cyanidin-based anthocyanins. This cultivar therefore contains a carnation F3'H and DFR activity that an introduced F3'5'H would need to compete with for substrate. The carnation cultivar Monte Lisa produces brick red colored flowers that normally accumulate pelargonidin-based anthocyanins. This cultivar is thought to lack fully functional F3'H activity and contain a DFR that is capable of acting on DHK and thus an introduced F3'5'H would only be required to compete with the endogenous DFR for substrate.

TABLE 11 Results of transgenic analysis of petals from carnations transformed with T-DNAs containing *F3'5'H* gene expression cassettes (*AmCHS 5': F3'5'H: petD8 3'*).

F3'5'H	pCGP	cv.	#tg	TLC+	HPLC+	Highest % del	Av. % del	Northern+
Salvia#2	2121	KC	22	2/16	3/4	12.5%	7%	*nd*
		ML	21	17/18	9/9	76%	57%	14/15
Salvia#47	2122	KC	23	6/12	8/8	29%	12%	*nd*
		ML	25	21/22	17/17	88%	56%	12/14
Sollya	2130	KC	30	22/27	17/17	35%	11%	*nd*
		ML	23	14/15	14/14	76%	49%	13/14
Butterfly pea	2135	KC	22	0/16	0/1	*nd*	*nd*	*nd*
		ML	24	19/20	13/13	23%	10%	14/14
Gentian	1498	KC	22	0/14	*nd*	*nd*	*nd*	7/8
		ML	2	2/2	1/1	*nd*	*nd*	1/2
pansy BP#18	1972	KC	26	18/20	12/12	14%	9%	19/19
		ML	21	15/16	8/8	80%	66%	14/16
pansy BP#40	1973	KC	26	11/15	7/8	18%	8%	13/17
		ML	33	19/22	20/20	72%	52%	12/15
petunia *petHf1*	1452	KC	104	41/64	*nd*	3,5%	1.3%	15/17
		ML	48	39/41	26/26	75%	30%	12/13
petunia *petHf2*	1524	ML	27	18/19	17/17	81%	41%	12/14

F3'5'H = *F3'5'H* sequence contained on the T-DNA

pCGP =plasmid pCGP number of the binary vector used in the transformation experiment

cv. = cultivar

KC = carnation cultivar Kortina Chanel (cyanidin line)

ML = carnation cultivar Monte Lisa (pelargonidin line)

#tg = total number of transgenics produced

TLC+ = number of individual events in which delphinidin or delphinidin-based molecules was detected in petals (as determined by TLC) over the total number of individual events analyzed

HPLC+ = number of individual events in which delphinidin or delphinidin-based molecules was detected in petals (as determined by HPLC) over the total number of individual events analyzed

Highest % del = Highest % delphinidin or delphinidin-based molecules detected in the petals for the population of transgenic events

Av % del = average % delphinidin or delphinidin-based molecules detected in the petals for the population of transgenic events

Northern = number of individual events in which the specific intact *F3'5H* transcripts were detected by Northern blot analysis in total RNA isolated from petals over the total number of events analyzed

nd = not done

The results suggest that all of the *F3'5'H* sequences evaluated (petunia *petHf1*, petunia *petHf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40* and Gentian *Gen#48*) were stable in carnation and resulted in the production of novel delphinidin-based pigments in carnation flowers. Intact transcripts of each *F3'5'H* were detected by Northern blot analysis in total RNA isolated from petals of the transgenic carnations.

Petunia

The *F3'5'H* genes described in Example 7 were evaluated for their ability to lead to the production of delphinidin-based pigments in petunia petals. The *P. hybrida* F1 hybrid Skr4 x SW63 which is homozygous recessive for *Hf1* and *Hf2*, was used in the transformation experiments. Although Skr4 x SW63 is homozygous recessive for *Hf1* and *Hf2*, these mutations do not completely block production of the endogenous F3'5'H (see US Patent Number 5,349,125) and low levels of malvidin are produced to give the petal limb a pale lilac color. Malvidin is the methylated derivative of the 3'5'-hydroxylated pigment, delphinidin or delphinidin-based molecules (Figures 1A and 1B). Spectrophotometric

analysis was used as a measure of total anthocyanins accumulating in petals from the transgenic petunia flowers. The increased level of anthocyanins and/or the color change detected was used as a guide to the efficacy of the *F3'5'H* gene under evaluation.

TABLE 12 Results of transgenic analysis of petals from *P. hybrida* cv Skr4 x SW63 plants transformed with T-DNAs containing *F3'5'H* gene expression cassettes (*AmCHS 5'*: *F3'5'H*: *petD8 3'*).

F3'5'H	pCGP	# tg	TLC+	Col	↑A/c	Best	Av.	Northern+	Best color
control	*na*	*na*	*na*	*na*	*na*	144-250		0	75C
Gentian#48	1498	22	3/5	18/20	*nd*			6/6	72B/78A
Butterfly pea	2135	24	18/20	22/24	23/24	4427	2397	*nd*	74A/78A
Kennedia	2256	24	22/24	22/24	22/24	4212	2592	*nd*	74A/78A
Salvia#2	2121	24	21/24	21/24	21/24	2471	1730	*nd*	78A
Salvia#47	2122	19	17/19	16/19	16/19	2634	1755	*nd*	78A/80A
Sollya#5	2130	22	14/16	13/16	13/16	3446	1565	*nd*	78A
pansy BP#18	1972	22	*nd*	20/22	*nd*	*nd*	*nd*	9/9	74A/B
pansy BP#40	1973	19	8/8	18/19	18/20	2583	1556	*nd*	74/78A
petunia *petHf1*	484	16	*nd*	9/16	8/15	2683	1250	*nd*	74A/B
petunia *petHf2*	1524	20	*nd*	18/20	8/8	4578	2357	8/8	74A/B

F3'5'H = *F3'5'H* sequence contained on the T-DNA

pCGP = plasmid pCGP number of the binary vector used in the transformation experiment

#tg = total number of transgenics produced

TLC+ = number of individual events in which malvidin was detected in the flowers (at a level above the Skr4 x Sw63 background) (as determined by TLC) over the total number of individual events analyzed

Col = number of individual events that produced flowers with an altered flower color compared to the control over the total number examined

↑ A/c = number of individual events that had an increased level of anthocyanins in petals as measured by spectrophotometric analysis of crude extracts over the number of individual events analyzed (in μmoles/g)

Best = highest anthocyanin amount as measured by spectrophotometric analysis of crude extracts from a flower of an individual event (in μmoles/g)

Av = the average amount of anthocyanin detected as measured by spectrophotometric analysis of crude extracts from a flower in the population of transgenic flowers analysed (in μmoles/g)

Northern = number of individual events in which the specific intact *F3'5H* transcripts were detected by Northern blot analysis in total RNA isolated from petals over the total number of events analyzed

Best color = most dramatic color change recorded for the transgenic population

nd = not done

na = not applicable

Introduction of the *F3'5'H* gene expression cassettes into Skr4 x SW63 led to a dramatic flower color change from pale lilac to purple with a dramatic increase in the production of malvidin in the petals..

The results suggest that all of the *F3'5'H* sequences tested (petunia *petHf1*, petunia *petHf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40*, Gentian *Gen#48*, Kennedia *Kenn#31*) were stable in petunia petals and resulted in the complementation of the *Hf1* or *Hf2* mutation in the Skr4 x SW63 petunia line leading to dramatically increased levels of malvidin accumulation with a concomitant color change

Rose

The *F3'5'H* genes described in Example 7 were evaluated for their ability to lead to the production of delphinidin-based pigments in rose petals. A selection of three rose cultivars, Kardinal (Kard), Soft Promise (SP) or Lavande (Lav) were used in transformation experiments. The rose cultivar Kardinal produces red colored flowers that normally accumulate cyanidin-based anthocyanins. This cultivar therefore contains rose F3'H and DFR activities that the introduced F3'5'H would need to compete with for substrate. The rose cultivar Lavande produces light pink colored flowers that normally accumulate cyanidin-based anthocyanins. This cultivar therefore contains functional rose F3'H and DFR activities that the introduced F3'5'H would need to compete with for substrate. The rose cultivar Soft Promise produces apricot colored flowers that normally accumulate pelargonidin. This cultivar is thought to lack a fully functional rose F3'H activity and contain a DFR that is capable of acting on DHK and thus the introduced F3'5'H would only be required to compete with the endogenous rose DFR for substrate.

TABLE 13 Results of transgenic analysis of petals from roses transformed with T-DNAs containing *F3'5'H* gene expression cassettes (*CaMV 35S: F3'5'H: ocs 3'*).

F3'5'H	plasmid	Cult	#tg	TLC +	HPLC+	Highest % del	Av. % del	Northern+
Salvia2	pCGP2120	Kard	30	18/20	21/21	12%	5%	18/18
Salvia47	pCGP2119	Kard	22	11/16	9/9	7.1%	2%	12/15
Sollya	pCGP2131	Kard	27	0/23	2/2	1%	0.5%	6/6
Butterfly	pCGP2134	Kard	29	0/15	*nd*	*na*	*na*	0/9
pea	pBEBF5	Lav	25	*nd*	0/25	0%	0%	*nd*
Gentian	pCGP1482	Kard	27	0/23	*nd*	*na*	*na*	0/23
	pBEGHF48	Lav	23	*nd*	0/23	0%	0%	0/23
pansy BP18	pCGP1967	Kard	56	30/33	33/34	58%	12%	21/21
		SP	36	21/24	18/18	65%	35%	16/21
pansy BP40	pCGP1969	Kard	22	15/15	15/15	24%	9%	16/16
		SP	37	17/17	16/17	80%	54%	11/13

F3'5'H	plasmid	Cult	#tg	TLC+	HPLC+	Highest % del	Av. % del	Northern+
Petunia	pCGP1638	Kard	22	0/21	nd	na	na	0/16
petHf1	pCGP1392	Lav.	34	nd	0/34	0%	0%	*nd*
Petunia *petHf2*	pCGP2123	Kard	41	0/26	nd	na	na	0/10
Lavender	pBELF8	Lav	28	*nd*	4/28	4%	3.5%	*nd*

F3'5'H = the *F3'5'H* sequence contained on the T-DNA

plasmid = the plasmid number of the binary vector used in the transformation experiment

Cult = *Rosa hybrida* cultivar

Kard = Kardinal

SP = Soft Promise

Lav = Lavande

#tg = # of independent transgenic events produced

TLC+ = number of individual events that accumulated detectable delphinidin or delphinidin-based molecules (as determined by TLC) in the petals over the number of individual events analyzed

HPLC+ = number of individual events that accumulated detectable delphinidin or delphinidin-based molecules (as determined by HPLC) in the petals over the number of individual events analyzed

Northern = number of individual events in which the specific intact *F3'5H* transcripts were detected by Northern blot analysis in total RNA isolated from petals over the total number of events analyzed

nd = not done

The results suggest surprisingly that not all of the *F3'5'H* sequences assessed (petunia *petHf1*, petunia *petHf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40*, Gentian *Gen#48*, Kennedia *Kenn#31* and Lavender *LBG*) were functional in rose. In fact transcripts of the introduced *F3'5'H* sequences isolated from *Clitoria ternatea* (butterfly pea), *Gentiana triflora*, (gentian) and *Petunia hybrida* (petunia) failed to accumulate in rose petals. Only full-length *F3'5'H* transcripts from pansy, salvia, kennedia, sollya and lavender accumulated in rose petals. However although Kennedia *F3'5'H* transcripts did accumulate in rose petals, there was either no accumulation of the enzyme or the enzyme produced was either not functional or was unable to compete with the endogenous rose F3'H and DFR enzymes to allow for the production of delphinidin or delphinidin-based molecules pigments. Of the *F3'5'H* sequences evaluated, only the *F3'5'H* sequences derived from cDNA clones from *Salvia spp.* (*Sal#2* and *Sal#47*), *Viola spp.* (*BP#18* and *BP#40*), *Sollya spp.* (*Sol#5*) and *Lavandula nil* (*LBG*) resulted in the production of delphinidin or delphinidin-based molecules based pigments in rose petals. Based on the relative percentages of delphinidin or delphinidin-based molecules produced in rose petals, the *F3'5'H* sequences from pansy (*BP#18* and *BP#40*) were revealed to be the most effective of those assessed at producing delphinidin or delphinidin-based molecules in rose petals.

Introduction of Viola spp. F3'5'H sequence into Rosa hybrida cv. Medeo and Pamela

As described in the introduction, copigmentation with other flavonoids, further modification of the anthocyanidin molecule and the pH of the vacuole impact on the color produced by anthocyanins. Therefore, selection of rose cultivars with relatively high levels of flavonols and relatively high vacuolar pH would result in bluer flower colors upon production of delphinidin or delphinidin-based molecules pigments.

The rose cultivar Medeo generally produces cream-colored to pale apricot flowers (RHSCC 158C to 159A). HPLC analysis of the anthocyanidins and flavonols accumulating in Medeo rose petals revealed that the petals accumulate high levels of flavonols (2.32 mg/g kaempferol, 0.03 mg/g quercetin) and very low levels of anthocyanins (0.004 mg/g

cyanidin, 0.004 mg/g pelargonidin). The estimated vacuolar pH of Medeo petals is around 4.6.

The rose cultivar Pamela produces white to very pale pink colored flowers. It similarly accumulates low levels of anthocyanin and relatively high levels of flavonols.

The T-DNA contained in the construct pCGP1969 (Figure 30) incorporating the pansy *F3'5'H* clone, *BP#40*, was also introduced into the rose cultivars Medeo and Pamela resulting in the production of over 90% delphinidin or delphinidin-based molecules in these roses and leading to a dramatic color change and novel colored flowers. The most dramatic color change in transgenic Medeo flowers was to a purple/violet color of RHSCC 70b, 70c, 80c, 186b. The most dramatic color change in transgenic Pamela flowers was to a purple/violet color of RHSCC 71c, 60c, 71a, 80b.

In conclusion, two unexpected findings were revealed when gene sequences that had been proven to lead to functionality in petunia and carnation were introduced into roses.

First, the petunia *F3'5'H petHf1* (and *petHf2*) sequences that had resulted in novel color production in carnation and also proven to lead to synthesis of a functional enzyme in petunia did not lead to full-length (or intact) transcript accumulation (as detectable by Northern blot analysis) in rose petals. In fact, there was either no accumulation of full-length or intact transcript or the transcripts that were detected were degraded and were seen as low MW (or fast migrating) smears on RNA blots indicating the presence of low MW heterologous hybridizing RNA. Therefore in order to find a *F3'5'H* sequence that would accumulate in rose and lead to a functional enzyme, a number of *F3'5'H* sequences were isolated. Again it was not obvious which sequence would lead to an active enzyme in rose petals. All of the *F3'5'H* sequences isolated were tested for functionality in carnation and/or petunia and all led to accumulation of intact transcripts and production of a functional *F3'5'H* activity. However only *F3'5'H* sequences from pansy (*BP#18* and *BP#40*), salvia (*Sal#2* and *Sal#47*), sollya (*Soll#5*), kennedia (*Kenn#31*) and lavender (*LBG*) resulted in accumulation of intact full-length transcripts and only those from pansy

(*BP#18* and *BP#40*), salvia (*Sal#2* and *Sal#47*), sollya (*Soll#5*) and lavender (*LBG*) resulted in production of a functional enzyme in rose as measured by the synthesis of delphinidin or delphinidin-based molecules.

Secondly that it was not obvious which promoters would be effective in rose. Promoter cassettes that had been tested and proven to be functional in carnation and petunia flowers did not lead to accumulation of detectable transcripts in rose petals. Of the promoters tested in rose, only *CaMV 35S*, *RoseCHS 5'*, *ChrysCHS 5'*, *mas 5'* and *nos 5'* promoters led to intact and detectable *GUS* or *nptII* or *SuRB* transcript accumulation in rose petals.

Table 14 shows a summary of the results obtained when assessing F3'5'H sequences from various species in petunia, carnation and rose.

TABLE 14 Summary of effectiveness of the *F3'5'H* sequences in petunia, carnation and rose

F3'5'H	Petunia		Carnation		Rose	
	Mal	RNA	Del	RNA	Del	RNA
Kennedia (*Kenn#31*)	+	*nd*	*nd*	*nd*	-	+
Gentian (*Gen#48*)	+	+	+	+	-	-
Salvia (*Sal#2*)	+	*nd*	+	+	+	+
Salvia (*Sal#47*)	+	*nd*	+	+	+	+
Sollya (*Sol#5*)	+	*nd*	+	+	+	+
Butterfly pea (*BpeaHF2*)	+	*nd*	+	+	-	-
Pansy (*BP#18*)	+	+	+	+	+	+
Pansy (*BP#40*)	+	*nd*	+	+	+	+
Petunia (*petHf1*)	+	+	+	+	-	-
Petunia (*petHf2*)	+	+	+	+	-	-
Lavender (*LBG*)	*nd*	*nd*	*nd*	*nd*	*nd*	+

nd = not done

Mal = malvidin detected in petals as analysed by TLC

Del = delphinidin or delphinidin-based molecules detected in petals as analysed by TLC or HPLC

\+ = yes

\- = no

EXAMPLE 9

Use of pansy F3'5'H sequences in species other than rose

Gerbera

From the examples above, it was clear that the pansy *F3'5'H* sequences, *BP#18* and *BP#40*, resulted in functional F3'5'H activity and lead to the production of high levels of delphinidin or delphinidin-based molecules in roses and carnations.

The T-DNA from binary construct pCGP1969 (described in Example 8) (Figure 30) containing the chimeric *CaMV 35S: pansy BP#40 F3'5'H: ocs 3'* gene expression cassette was introduced into the gerbera cultivar Boogie *via Agrobacterium*-mediated transformation, to test the functionality of the pansy *F3'5'H* sequence in gerbera.

Of six events produced to date, one (#23407) has produced flowers with a dramatic color change (RHSCC 70c) compared to the control flower color (RHSCC 38a, 38c).

The color change of the petals of the transgenic gerbera has been correlated with the presence of delphinidin or delphinidin-based molecules as detected by TLC.

Other species

In order to produce delphinidin or delphinidin-based molecules pigments in plants that do not normally produce delphinidin-based pigments and does not contain a flavonoid 3'5'-hydroxylase constructs containing a *F3'5'H* gene (such as but not limited to a chimaeric *Viola spp.* and/or *Salvia spp.* and/or *Sollya spp.* and/or *Lavandula spp.* and/or *Kennedia spp. F3'5'H* gene) are introduced into a species that does not normally produce

delphinidin-based pigments. Such plants may include but are not limited to carnation, chrysanthemum, gerbera, orchids, *Euphorbia*, *Begonia* and *apple*.

EXAMPLE 10

Characteristics of F3'5'H sequences evaluated in petunia, carnation and rose

Gene regulation in eukaryotes is, in simple terms, facilitated by a number of factors which interact with a range of sequences proximal and distal to a nucleotide sequence coding for a given polypeptide. Engineering expression cassettes for introduction into plants for the generation of one or more traits is based on an understanding of gene regulation in eukaryotes in general and, in selected cases, plants in particular. The essential elements include a series of transcriptional regulation sequences typically, but not exclusively, located upstream or 5' to the point of transcription initiation. Such elements are typically described as enhancers and promoters, the latter being proximal to the point of transcription initiation. Immediately downstream from, or 3' to, the initiation of transcription point is a variable region of transcribed DNA which is denoted as the 5' untranslated region (5'utr) which plays a role in transcript stability and translational efficiency. Such sequences, when engineered into expression cassettes, are frequently chimeric and may be derived from sequences naturally occurring adjacent to the coding sequence and/or adjacent to a given promoter sequence. The coding sequence (sometimes disrupted by introns) lies 3' to the 5'utr followed by a 3'utr important to transcript (mRNA) stability and translational efficiency. Sequences 3' to the end of the coding region and 3' to the 3'utr itself are denoted as terminator sequences. All these elements make up an expression cassette. In making direct comparisons between promoters or other elements it is important to maintain uniformity in the remaining elements of an expression cassette. Hence, when comparing the efficacy of various F3'5'H sequences it was possible to confine the sequences leading to instability and the subsequent autodegradation of engineered mRNA and resultant absence of tri-hydroxylated products (delphinidin or delphinidin-based molecules derivatives) to the region coding for the F3'5'H and not to other elements in the expression cassette such as 5' utr and/or 3'utr sequences for example.

In an attempt to identify motifs or similarities between the *F3'5'H* sequences that resulted in full-length transcripts being detected in total RNA isolated from rose flowers, and ultimately delphinidin or delphinidin-based molecules production, comparisons across a range of parameters were performed. These included sequence identities at nucleic acid and amino acid levels, sequence alignments, taxonomic classifications, % of A or T nucleotides present in the sequence, % of codons with an A or T in the third position etc.

Taxonomic classification

The taxonomy of each species from which the *F3'5'H* sequences were isolated was examined (Table 15). There appeared to be no obvious link between the subclass classification and whether the *F3'5'H* sequence resulted in an intact transcript and subsequent delphinidin or delphinidin-based molecules production in roses.

Table 15: Taxonomic classifications of the species that *F3'5'H* sequences were isolated from and whether the use of the sequences resulted in intact transcript in rose petals that were detectable by RNA blot analysis.

Flower	Species	Family	Order	Subclass	Intact transcript	Delphinidin in rose petals
gentian	*Gentiana triflora*	Gentianaceae	Gentianales	Asteridae	NO	NO
lavender	*Lavandula nil*	Lamiaceae	Lamiales	Asteridae	YES	YES
salvia	*Salvia spp.*	Lamiaceae	Lamiales	Asteridae	YES	YES
sollya	*Sollya spp.*	Pittosporaceae	Apiales	Asteridae	YES	YES
petunia	*Petunia hybrida*	Solanaceae	Solanales	Asteridae	NO	NO
kennedia	*Kennedia spp.*	Fabaceae	Fabales	Rosidae	YES	NO
butterfly pea	*Clitoria ternatea*	Fabaceae	Fabales	Rosidae	NO	NO
pansy	*Viola spp.*	Violaceae	Malpighiales	Rosidae	YES	YES
rose	*Rosa hybrida*	Rosaciae	Rosales	Rosidae	na	na

Intact transcript = full-length *F3'5'H* mRNA detected by Northern blot analysis in total RNA isolated from petals from transgenic roses

Comparison of F3'5'H nucleotide sequences

The nucleotide sequence identities between each of the *F3'5'H* sequences evaluated were determined using the ClustalW program (Thompson *et al.*, 1994, *supra*) within the MacVector™ version 6.5.3 application program (Oxford Molecular Ltd., England) (Table 16). There were no obvious differences between the *F3'5'H* sequences that resulted in the detection of intact full-length transcripts in RNA isolated from rose petals and those that didn't.

Table 16: Percentage of nucleic acid sequence identity between the nucleotide sequences of the *F3'5'H* isolated from various species. *F3'5'H* sequences that resulted in intact transcripts being detected in RNA isolated from rose petals are underlined and in italics.

	BP18	*BP40*	*Lav*	*Sal47*	*Sal2*	*Sol1*	*Kenn*	Bpea	Gent	PetHf1	PetHf2
BP18	100	82	60	61	62	51	60	62	62	59	62
BP40		100	60	57	58	50	59	62	58	60	62
Lav			100	68	68	48	57	57	58	59	60
Sal47				100	95	48	56	57	59	57	58
Sal2					100	49	57	58	60	57	59
Sol1						100	48	50	50	48	51
Kenn							100	70	56	64	60
Bpea								100	59	59	62
Gent									100	61	64
PetHf1										100	84
PetHf2											100

Comparison of F3'5'H translated nucleotide sequences

The translated nucleotide sequence identities and similarities between each of the *F3'5'H* sequences evaluated were also determined using the ClustalW program (Thompson *et al.*, 1994, *supra*) within the MacVector™ version 6.5.3 application program (Oxford Molecular Ltd., England) (Table 17). There were no obvious differences between the *F3'5'H* sequences that resulted in the detection of intact full-length transcripts in RNA isolated from rose petals and those that didn't.

Table 17: Percentage of the amino acid sequence identity and similarity (in brackets) between F3'5'H sequences isolated from various species. F3'5'H sequences that resulted in intact transcripts being detected in RNA isolated from rose petals are underlined and in italics.

	BP18	*BP40*	*Lav*	*Sal47*	*Sal2*	*Sol1*	*Kenn*	Bpea	Gent	PetHf1	PetHf2
BP18	100	91 (94)	65 (77)	65 (76)	65 (76)	44 (63)	69 (83)	64 (75)	69 (80)	74 (85)	74 (85)
BP40		100	67 (89)	66 (77)	66 (77)	46 (64)	69 (82)	64 (75)	68 (79)	74 (85)	75 (86)
Lav			100	75 (86)	75 (86)	45 (63)	63 (79)	59 (74)	66 (80)	68 (82)	69 (83)
Sal47				100	98	45 (65)	64 (78)	60 (72)	64 (76)	68 (79)	69 (81)
Sal2					100	45 (65)	64 (78)	60 (72)	63 (75)	68 (79)	69 (81)
Sol1						100	46 (66)	41 (61)	44 (62)	46 (67)	46 (66)
Kenn							100	72 (80)	65 (75)	71 (83)	72 (83)
Bpea								100	69 (81)	65 (75)	65 (74)
Gent									100	73 (82)	73 (82)
PetHf 1										100	93 (95)
PetHf 2											100

Perceniage of nucleotides A or T in the F3'5'H DNA sequences

There is some evidence to suggest that the choice of codons influences the rate of translation and mRNA degradation. Certain codons are used less frequently than others are and this may be related to the abundance of isoaccepting tRNAs. Transfer RNAs corresponding to rare codons are less abundant in *E.coli* and yeast than tRNAs corresponding to preferred codons (van Hoof and Green, *Plant Molecular Biology*, 35: 383-387, 1997). Examples of altering codon usage and making a gene more "plant-like" are the bacterial *B.t.* toxin gene (reviewed in Diehn *et al.*, *Genet Engin*, 18: 83-99, 1996) and the jellyfish *gfp* gene (Haseloff et al., *Proc. Natl. Acad. Sci USA*, 94: 2122-2127, 1997). However as commented in van Hoof and Green, (1997) (*supra*), the effect of eliminating the rare codons in the *B.t.* genes increased the GC content, thereby eliminating AU-rich sequences that may be responsible for improper recognition of introns and polyadenylation sites as well as removing instability determinants. Alteration of codon usage in the jellyfish *gfp* gene also resulted in removal of a cryptic intron (Haseloff *et al.*, 1996, *supra*). Studies examining the effect of codon usage and instability elements have generally been limited to differences between genes isolated from species in different kingdoms ie. bacterial versus yeast versus animal versus plant. Within the plant kingdom, differences have been observed between the dicotyledons and the monocotyledons. Studies on transgenic plants have suggested that promoter fragments used to drive gene expression in dicotyledonous plants are not as effective when used in monocotyledonous plants (see Galun and Breiman, Transgenic Plants, Imperial College Press, London, England, 1997). Differences in the methylation and ultimate expression of a *DFR* transgene in *Petunia hybrida* (dicot) were detected when a maize (monocot) *DFR* cDNA was compared with a gerbera (dicot) *DFR* cDNA (Elomaa *et al.*, *Molecular and General Genetics*, 248: 649-656, 1995). The conclusion was that the gerbera *DFR* cDNA had a higher AT content (lower GC content) and was more "compatible" with the genomic organization of petunia preventing it being recognised as a foreign gene and hence silenced by methylation. (Rose along with carnation and petunia are dicotyledons and the *F3'5'H* genes tested were all isolated from dicotyledonous plants.) These points serve to illustrate that degradation and stability mechanisms are not understood in detail and differences appear between plants and other kingdoms and within the plant kingdom.

The content of A and T was examined in the *F3'5'H* cDNAs evaluated along with that of four flavonoid pathway genes (F3'H, DFR, CHS, FLS) that had been isolated from rose (Table 18). The third position of each codon (within the open reading frame) was also examined and the percentage of codons with an A or a T in the third position was calculated (Table 18).

Table 18: Summary of the percentage amount of A or T dinucleotides in the F3'5'H sequences isolated and whether the F3'5'H resulted in full-length transcripts being detected in rose petals by Northern blot analysis.

F3'5'H seq	%AT	% A or T in 3rd	RNA	Delphinidin
Viola BP#18	50	40	YES	YES
Viola BP#40	51	35	YES	YES
Salvia#2	48	33	YES	YES
Salvia#47	48	34	YES	YES
Sollya#5	54	54	YES	YES
LavenderLBG	50	37	YES*	YES
Kennedia#31	54	47	YES	NO
petunia *petHf1*	61	66	NO	NO
petunia *petHf2*	59	65	NO	NO
Gentian#48	57	57	NO	NO
Butterfly pea#*HF2*	57	53	NO	NO
rose *F3'H*	47	34	**	na
rose *CHS*	52	42	**	na

F3'5'H seq	%AT	% A or T in 3rd	RNA	Delphinidin
rose *DFR*	53	46	**	na
rose *FLS*	56	43	**	na

%AT = % of nucleotides that are A or T in the nucleic acid sequence

%A or T in 3rd = the percentage of codons that have an A or T in the third position

RNA = whether a full-length mRNA transcript was detected by Northern blot analysis in total RNA isolated from rose petals

Del = whether any *delphinidin or delphinidin-based molecules* was detected by TLC or HPLC in rose petals

YES* =although Northern blot analysis of transgenic roses transformed with the lavender F3'5'H expression cassettes was not performed, it can be assumed that full-length transcript was produced since *delphinidin or delphinidin-based molecules* was detected in the rose petals.

rose F3'H (described in International Patent Application No. PCT/AU97/00124)

rose DFR (Tanaka et al., 1995, *supra*)

rose FLS (GenBank accession number AB038247)

rose CHS (GenBank accession number AB038246)

The AT content of the four rose sequences (above) encoding flavonoid pathway enzymes had an AT content of between 47 and 56%. In general the AT content of the *F3'5'H* sequences that resulted in intact transcripts in rose petals was between 48 and 54%. However the *F3'5'H* sequences that did not result in intact transcripts accumulating in rose petals generally had a higher AT content of between 57 and 61%. Hence the AT content of the introduced *F3'5'H* genes into rose may be a factor in whether an intact transcript accumulates in rose petals and so leads to production of F3'5'H and *delphinidin or delphinidin-based molecules*.

The nucleotide base at the third position of each codon of the four rose sequences encoding flavonoid pathway enzymes was generally an A or a T in 34 to 46% of the codons. In general *F3'5'H* sequences that resulted in intact transcripts in rose petals contained an A or a T in the third position of each codon in 33 to 54% of the codons. However the *F3'5'H* sequences that did not result in intact transcripts accumulating in rose petals generally contained an A or a T in the third position of each codon in 53 to 66% of the codons. So the percentage of codons with an A or a T in the third position of the introduced *F3'5'H* genes into rose may also be a factor in whether an intact transcript is accumulates in rose petals and so leads to production of F3'5'H and *delphinidin or delphinidin-based molecules*.

It may be that by altering the overall content of the nucleotides A and/or T in any *F3'5'H* DNA sequence that does not result in an intact transcript in rose such as but nor limited to the *Petunia hybrida petHf1*, *Petunia hybrida petHf2*, *Clitora ternatea* (butterfly pea) *BpeaHF2* or *Gentiana triflora* (gentian) *Gen#48*, to a level more consistent with that found in rose genes, intact transcripts will accumulate and result in the efficient translation of *F3'5'H* transcripts and so to *delphinidin or delphinidin-based molecules* accumulation in rose petals. One way of altering the AT content of the DNA sequence without altering the amino acid sequence is to target the degeneracy of the third position of each codon.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a flavonoid 3', 5' hydroxylase (F3'5'H) or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in detectable levels of *delphinidin or delphinidin-based molecules* as measured by a chromatographic technique.

2. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in a sufficient level and length of transcript which is translated to said F3'5'H as determined by detectable levels of *delphinidin or delphinidin-based molecules* as measured by a chromatographic technique.

3. The isolated nucleic acid molecule of claim 1 or 2 wherein expression of said nucleic acid molecule in said rose petal results in a visually detectable colour change.

4. The isolated nucleic acid molecule of any one of claims 1to3, wherein the nucleic acid molecule is derived from a plant selected from the list comprising a *Viola spp.*, *Salvia spp.*, *Sollya spp.*, *Lavandula spp.* and *Kennedia spp.*

5. The isolated nucleic acid molecule of claim 4, wherein the nucleic acid molecule is derived from a *Viola spp.* plant.

6. The isolated nucleic acid molecule of claim 5, wherein the nucleic acid molecule is derived from the *Viola spp.*, cultivar Black Pansy.

7. The isolated nucleic acid molecule of any one of claims 5 or 6, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, an amino acid sequence having at least about 40% similarity to SEQ ID NO:10 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:12.

8. The isolated nucleic acid molecule of claim 7, comprising a nucleotide sequence selected from SEQ ID NO:9, SEQ ID NO:11, a nucleotide sequence having at least about 40% identity to SEQ ID NO:9, a nucleotide sequence having at least about 40% identity to SEQ ID NO:11, a nucleotide sequence capable of hybridizing to SEQ ID NO:9 or its complement under low stringency conditions and a nucleotide sequence capable of hybridizing to SEQ ID NO:11 or its complement under low stringency conditions.

9. The isolated nucleic acid molecule of claim 8, comprising the nucleotide sequence set forth in SEQ ID NO:9.

10. The isolated nucleic acid molecule of claim 8, comprising the nucleotide sequence set forth in SEQ ID NO:11.

11. The isolated nucleic acid molecule of claim 4, wherein the nucleic acid molecule is derived from *Salvia spp.*.

12. The isolated nucleic acid molecule of claim 11, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selected from SEQ ID NO:14, SEQ ID NO:16, an amino acid sequence having at least about 40% similarity to SEQ ID NO:14 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:16.

13. The isolated nucleic acid molecule of claim 12, comprising a nucleotide sequence selected from SEQ ID NO:13, SEQ ID NO:15, a nucleotide sequence having at least about 40% identity to SEQ ID NO:13, a nucleotide sequence having at least about 40% identity to SEQ ID NO:15, a nucleotide sequence capable of hybridizing to SEQ ID NO:13 or its complement under low stringency conditions and a nucleotide sequence capable of hybridizing to SEQ ID NO:15 or its complement under low stringency conditions.

14. The isolated nucleic acid molecule of claim 13, comprising the nucleotide sequence set forth in SEQ ID NO:13.

15. The isolated nucleic acid molecule of claim 13, comprising the nucleotide sequence set forth in SEQ ID NO:15.

16. The isolated nucleic acid molecule of claim 4, wherein the nucleic acid molecule is derived from *Sollya spp.*

17. The isolated nucleic acid molecule of claim 16, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selected from SEQ ID NO:18 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:18.

18. The isolated nucleic acid molecule of claim 17, comprising a nucleotide sequence selected from SEQ ID NO:17, a nucleotide sequence having at least about 40% identity to SEQ ID NO:17 and a nucleotide sequence capable of hybridizing to SEQ ID NO:17 or its complement under low stringency conditions.

19. The isolated nucleic acid molecule of claim 18, comprising the nucleotide sequence set forth in SEQ ID NO:17.

20. The isolated nucleic acid molecule of claim 4, wherein the nucleic acid molecule is derived from *Kennedia spp.*

21. The isolated nucleic acid molecule of claim 20, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selecting from SEQ ID NO:27 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:27.

22. The isolated nucleic acid molecule of claim 21, comprising a nucleotide sequence selected from SEQ ID NO:26, a nucleotide sequence having at least about 40% identity to SEQ ID NO:26 and a nucleotide sequence capable of hybridizing to SEQ ID NO:26 or its complement under low stringency conditions.

23. The isolated nucleic acid molecule of claim 22, comprising the nucleotide sequence set forth in SEQ ID NO:26.

24. The isolated nucleic acid molecule of claim 4, wherein the nucleic acid molecule is derived from *Lavandula spp.*

25. The isolated nucleic acid molecule of claim 24, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selected from SEQ ID NO:32 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:32.

26. The isolated nucleic acid molecule of claim 25, comprising a nucleotide sequence selected from SEQ ID NO:31, a nucleotide sequence having at least about 40% identity to SEQ ID NO:31 and a nucleotide sequence capable of hybridizing to SEQ ID NO:31 or its complement under low stringency conditions.

27. The isolated nucleic acid molecule of claim 26, comprising the nucleotide sequence set forth in SEQ ID NO:31.

28. The isolated nucleic acid molecule of any one of claims 1– to 4, wherein the nucleotide sequence comprises an overall percentage of less than or equal to 54% of the nucleotides

(i) A, or

(ii) T, or

(iii) A and T

in the third nucleotide position of each codon.

29. A construct comprising a sequence of nucleotides comprising:

(i) a promoter which is operable in rose petal tissue and wherein said promoter is operably linked to,

(ii) a nucleic acid molecule encoding F3'5'H, or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in detectable levels of *delphinidin or delphinidin-based molecules* as measured by a chromatographic technique and wherein said nucleic acid molecule is derived from a plant selected from the group consisting of a *Viola spp., Salvia spp., Sollya spp., Lavandula spp.* and *Kennedia spp.*

30. A construct comprising a sequence of nucleotides comprising:

(i) a promoter which is operable in rose petal tissue and wherein said promoter is operably linked to,

(ii) a nucleic acid molecule encoding F3'5'H, or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in a sufficient level and length of transcript which is translated to said F3'5'H as determined by detectable levels of *delphinidin or delphinidin-based molecules* as measured by a chromatographic technique.

31. The construct of claim 29 or 30 , wherein expression of said construct in said rose petal results in a visually detectable colour change.

32. The construct of any one of claims 29 to 31, wherein said promoter is selected from the group consisting of rose CHS, chrysanthemum CHS and CaMV 35S.

33. A construct of any one of claims 29 to 31 wherein said promoter comprises SEQ ID NO:5, or a functional equivalent thereof.

34. A construct of any one of claims 29 to 31 wherein said promoter comprises SEQ ID NO:30, or a functional equivalent thereof.

35. The construct of any one of claims 29 to 34 , wherein the nucleic acid molecule is derived from a *Viola spp*.

36. The isolated nucleic acid molecule of claim 35, wherein the nucleotide sequence encodes F3'5'H comprising an amino acid sequence selected from SEQ ID NO:10, SEQ ID NO:12, an amino acid sequence having at least about 40% similarity to SEQ ID NO:10 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:12.

37. The isolated nucleic acid molecule of claim 36, comprising a nucleotide sequence selected from SEQ ID NO:9, SEQ ID NO:11, a nucleotide sequence having at least about 40% identity to SEQ ID NO:9, a nucleotide sequence having at least about 40% identity to SEQ ID NO:11, a nucleotide sequence capable of hybridizing to SEQ ID NO:9 or its complement under low stringent conditions and a nucleotide sequence capable of hybridizing to SEQ ID NO:11 or is complement under low stringent conditions.

38. The isolated nucleic acid molecule of claim 37, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:9.

39. The isolated nucleic acid molecule of claim 37, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:11.

40. The isolated nucleic acid molecule of any one of claims 29 to 34, wherein the nucleic acid molecule is derived from *Salvia spp.*

41. The isolated nucleic acid molecule of claim 40, wherein the gene comprises a nucleotide sequence encoding F3'5'H comprising an amino acid sequence selected from SEQ ID NO:14, SEQ ID NO:16, an amino acid sequence having at least about 40% similarity to SEQ ID NO:14 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:16.

42. The isolated nucleic acid molecule of claim 41, wherein the gene comprises a nucleotide sequence selected from SEQ ID NO:13, SEQ ID NO:15, a nucleotide sequence having at least about 40% identity to SEQ ID NO:13, a nucleic sequence having at least about 40% identity to SEQ ID NO:15, a nucleotide sequence capable of hybridizing to SEQ ID NO:13 or its complements under low stringent conditions and a nucleotide sequence capable of hybridizing to SEQ ID NO:15 or is complement under low stringent conditions.

43. The isolated nucleic acid molecule of claim 42, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:13.

44. The isolated nucleic acid molecule of claim 42, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:15.

45. The isolated nucleic acid molecule of any one of claims 29 to 34, wherein the gene is derived from *Sollya spp.*

46. The isolated nucleic acid molecule of claim 45, wherein the gene encodes a F3'5'H comprising an amino acid sequence selected from SEQ ID NO:18 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:18.

47. The isolated nucleic acid molecule of claim 46, wherein the gene comprises a nucleotide sequence selected from SEQ ID NO:17, a nucleotide sequence having at least about 40% identity to SEQ ID NO:17 and a nucleotide sequence capable of hybridizing to SEQ ID NO:17 or its complements under low stringent.

48. The isolated nucleic acid molecule of claim 47, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:17.

49. The isolated nucleic acid molecule of any one of claims 29 to 34, wherein the gene is derived from *Kennedia spp.*

50. The isolated nucleic acid molecule of claim 49, wherein the gene encodes F3'5'H comprising an amino acid sequence selected from SEQ ID NO:27 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:27.

51. The isolated nucleic acid molecule of claim 50, wherein the gene comprises a nucleotide sequence selected from SEQ ID NO:26, a nucleotide sequence having at least about 40% identity to SEQ ID NO:26 and a nucleotide sequence capable of hybridizing to SEQ ID NO:26 or its complements under low stringent.

52. The isolated nucleic acid molecule of claim 51, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:26.

53. The isolated nucleic acid molecule of any one of claims 29 to 34, wherein the gene is derived from *Lavandula spp.*

54. The isolated nucleic acid molecule of claim 53, wherein the gene encodes F3'5'H comprising an amino acid sequence selected from SEQ ID NO:32 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:32.

55. The isolated nucleic acid molecule of claim 54, wherein the gene comprises a nucleotide sequence selected from SEQ ID NO:31 and a nucleotide sequence having at least about 40% identity to SEQ ID NO:31, a nucleotide sequence capable of hybridizing to SEQ ID NO:31 or its complements under low stringent conditions.

56. The isolated nucleic acid molecule of claim 55, wherein the gene comprises the nucleotide sequence set forth in SEQ ID NO:31.

57. A method for producing a transgenic flowering plant capable of synthesizing a F3'5'H, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence as defined in any one of claims 1 to 28, under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

58. A method for producing a transgenic plant with reduced indigenous or existing F3'5'H activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule as defined in any one of claims 1 to 28, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

59. A method for producing a genetically modified plant with reduced indigenous or existing F3'5'H activity, said method comprising altering the *F3'5'H* gene through modification of the indigenous sequences via homologous recombination from an appropriately altered *F3'5'H* gene as defined in any one of claims 1 to 28, or a derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

60. A method for producing a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence as defined in any one of claims 1 to 28, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

61. A method for producing a flowering plant exhibiting altered inflorescence properties, said method comprising alteration of a *F3'5'H* gene as defined in any one of claims 1 to 28, through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

62. A method for producing a transgenic plant capable of expressing a recombinant gene encoding F3'5'H as defined in any one of claims 1 to 28, or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of an mRNA molecule encoding said F3'5'H, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding F3'5'H, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell.

63. A genetically modified plant or part thereof or cells therefrom comprising an isolated nucleic acid molecule of any one of claims 1 to 28.

64. A genetically modified plant or part thereof or cells therefrom comprising an isolated nucleic acid molecule of any one of claims 1 to 28 or comprising a reduced level of expression of a nucleic acid molecule of any one of claims 1 to 28.

65. A genetically modified plant or part thereof or cells therefrom comprising an isolated nucleic acid molecule of any one of claims 1 to 28 or comprising an increased level of expression of a nucleic acid molecule of any one of claims 1 to 28.

66. The genetically modified plant or part thereof or cells therefrom any one of claims 63 to 65 , wherein the plant part is selected from sepal, bract, petiole, peduncle, ovaries, anthers, flowers, fruits, nuts, roots, stems, leaves, seeds.

67. The genetically modified plant or part thereof or cells therefrom of any one of claims 63 to 66, wherein the plant is a horticultural species, agricultural species or ornamental species.

68. Use of an isolated nucleic acid molecule as defined in any one of claims 1 to 28, in the manufacture of a genetic construct capable of expressing F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

69. A gene silencing construct comprising an isolated nucleic acid molecule as defined in any one of claims 1 to 28 or a complex thereof.

70. The genetically modified plant or part thereof or cells therefrom of 63 to 66, wherein the plant is selected from a rose, carnation, lisianthus, petunia, lily, pansy, gerbera, chrysanthemum, geranium, *Torenia, Begonia, Cyclamen, Nierembergia, Catharanthus, Pelargonium*, orchid, grape, apple, *Euphorbia* or *Fuchsia*.

71. An extract from a genetically modified plant or part thereof or cells therefrom from any one of claims 63 to 67 and 70.

72. The extract of claim 71, wherein the extract is a flavouring or food additive or health product or beverage or juice or colouring.

73. The method of any one of claims 57 to 62 wherein the genetically modified plant or part thereof or cells therefrom exhibit altered inflorescence.

74. An isolated recombinant F3'5'H. or peptide having F3'5'H activity encoded by a nucleic acid molecule as defined in any one of claims 1 to 28.

75. The isolated recombinant F3'5'H or peptide having F3'5'H activity of claim 74, wherein the recombinant F3'5'H or peptide having F3'5'H activity is a fusion molecule comprising two or more heterologous amino acid sequences.

76. An isolated recombinant F3'5'H or peptide having F3'5'H activity nucleic acid molecule of any one of claims 1 to 28 comprising a fusion of two or more heterologous nucleotide sequences.

77. A prokaryotic organism carrying a genetic sequence encoding a F3'5'H molecule according to any one of claims 1 to 28 extrachromasomally in plasmid form.

78. A eukaryotic organism carrying a genetic sequence encoding a F3'5'H molecule according to any one of claims 1 to 28 extrachromasomally in plasmid form.

79. The use of a nucleic acid molecule of any one of claims 1 to 28 in the manufacture of a genetically modified plant or part thereof or cells therefrom.

80. The genetically modified plant or part thereof or cells therefrom of claim 79, wherein the genetically modified plant or part thereof or cells therefrom exhibitsaltered flowers or inflorescence.

81. The use of a nucleic acid sequence as defined in any one of claims 1 to 28 in the manufacture of a genetic construct capable of expressing F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

82. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a F3'5'H or a polypeptide having F3'5'H activity, wherein said nucleic acid molecule is derived from butterfly pea.

83. The isolated nucleic acid molecule of claim 81, wherein the nucleotide sequence encodes a F3'5'H comprising an amino acid sequence selecting for SEQ ID NO:21 and an amino acid sequence having at least about 40% similarity to SEQ ID NO:21.

84. The isolated nucleic acid molecule of claim 83, comprising a nucleotide sequence selected from SEQ ID NO:20, a nucleotide sequence having at least about 40% identity to SEQ ID NO:20 and a nucleotide sequence capable of hybridizing to SEQ ID NO:20 or its complement under low stringency conditions.

85. The isolated nucleic acid molecule of claim 84, comprising the nucleotide sequence set forth in SEQ ID NO:20.

86. The isolated nucleic acid molecule of any one of claims 1 to 4, wherein the nucleotide sequence comprises an overall percentage of less than or equal to 55% of the nucleotides

(i) A, or
(ii) T, or
(iii) A and T.

87. An isolated nucleic acid molecule comprising SEQ ID NO:5 or a functional equivalent thereof.

88. An isolated nucleic acid molecule comprising SEQ ID NO:30 or a functional equivalent thereof.

89. An isolated nucleic acid molecule which has been modified so as to comprise a sequence of nucleotides encoding or complementary to a sequence encoding F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in detectable levels of ***delphinidin or delphinidin-based molecules*** as measured by a chromatographic technique wherein the nucleic acid molecule is derived from a plant selected from the list comprising ***Petunia spp.***, ***Gentiana spp.*** and *Clitoria spp.*

90. An isolated nucleic acid molecule which has been modified so as to comprise a sequence of nucleotides encoding or complementary to a sequence encoding F3'5'H or a polypeptide having F3'5'H activity wherein expression of said nucleic acid molecule in a rose petal tissue results in a sufficient level and length of transcript which is translated to said F3'5'H as determined by detectable levels of ***delphinidin or delphinidin-based molecules*** as measured by a chromatographic technique wherein the nucleic acid molecule is derived from a plant selected from the list comprising petunia, gentiana and butterfly pea.

91. The use of a nucleic acid sequence as defined in claim 89 or 90 in the manufacture of a genetic construct capable of expressing F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

92. The use of a nucleic acid sequence as defined in claim 89 or 90 in the manufacture of a genetically modified plant or part thereof or cells therefrom.

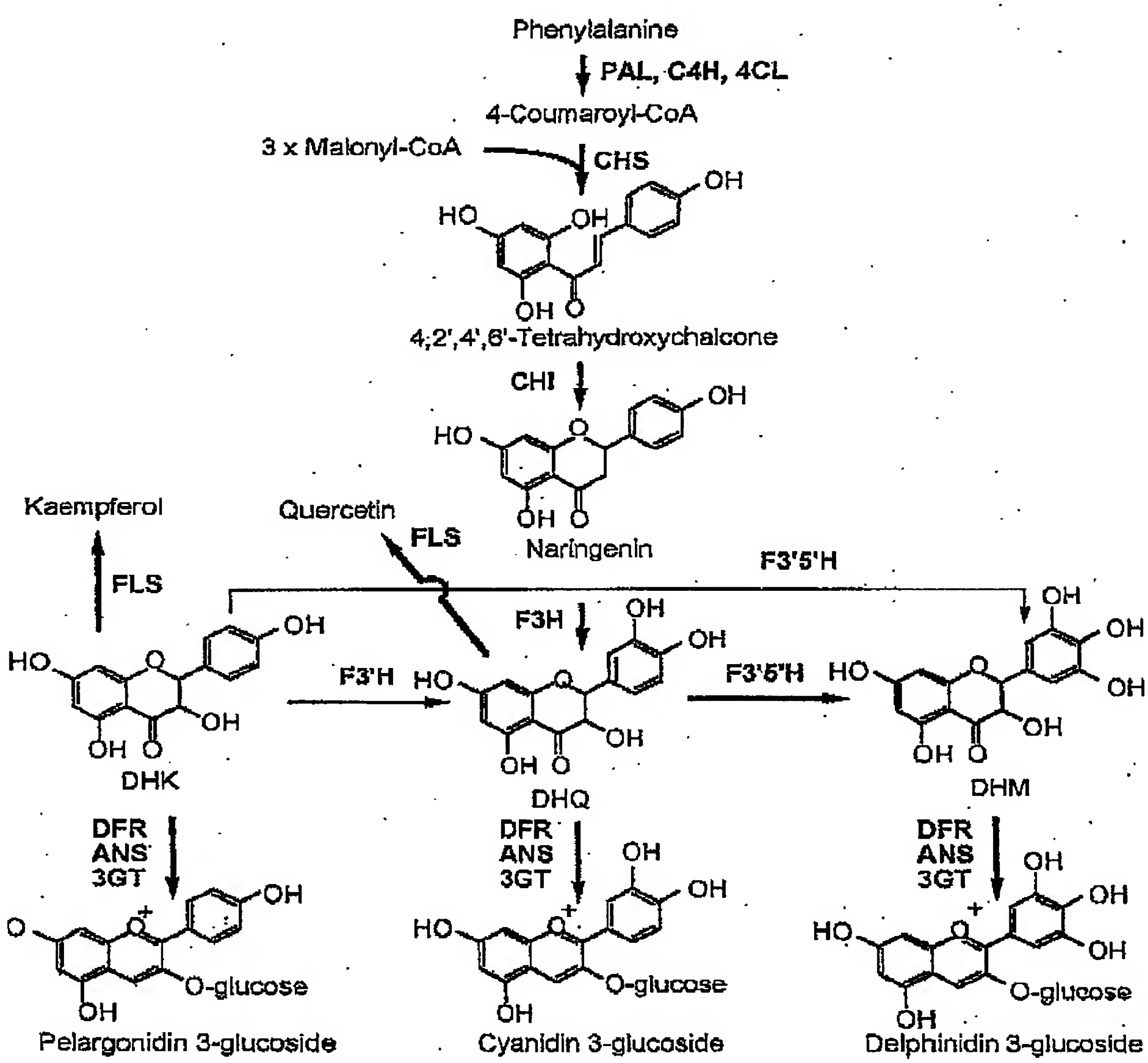

Phenylalanine
PAL, C4H, 4CL
4-Coumaroyl-CoA
3 x Malonyl-CoA
CHS
4,2',4',6'-Tetrahydroxychalcone
CHI
Naringenin
Kaempferol
Quercetin
FLS
FLS
F3'5'H
F3H
F3'H
F3'5'H
DHK
DHQ
DHM
DFR
ANS
3GT
DFR
ANS
3GT
DFR
ANS
3GT
O-glucose
O-glucose
O-glucose
Pelargonidin 3-glucoside
Cyanidin 3-glucoside
Delphinidin 3-glucoside

Figure 1a

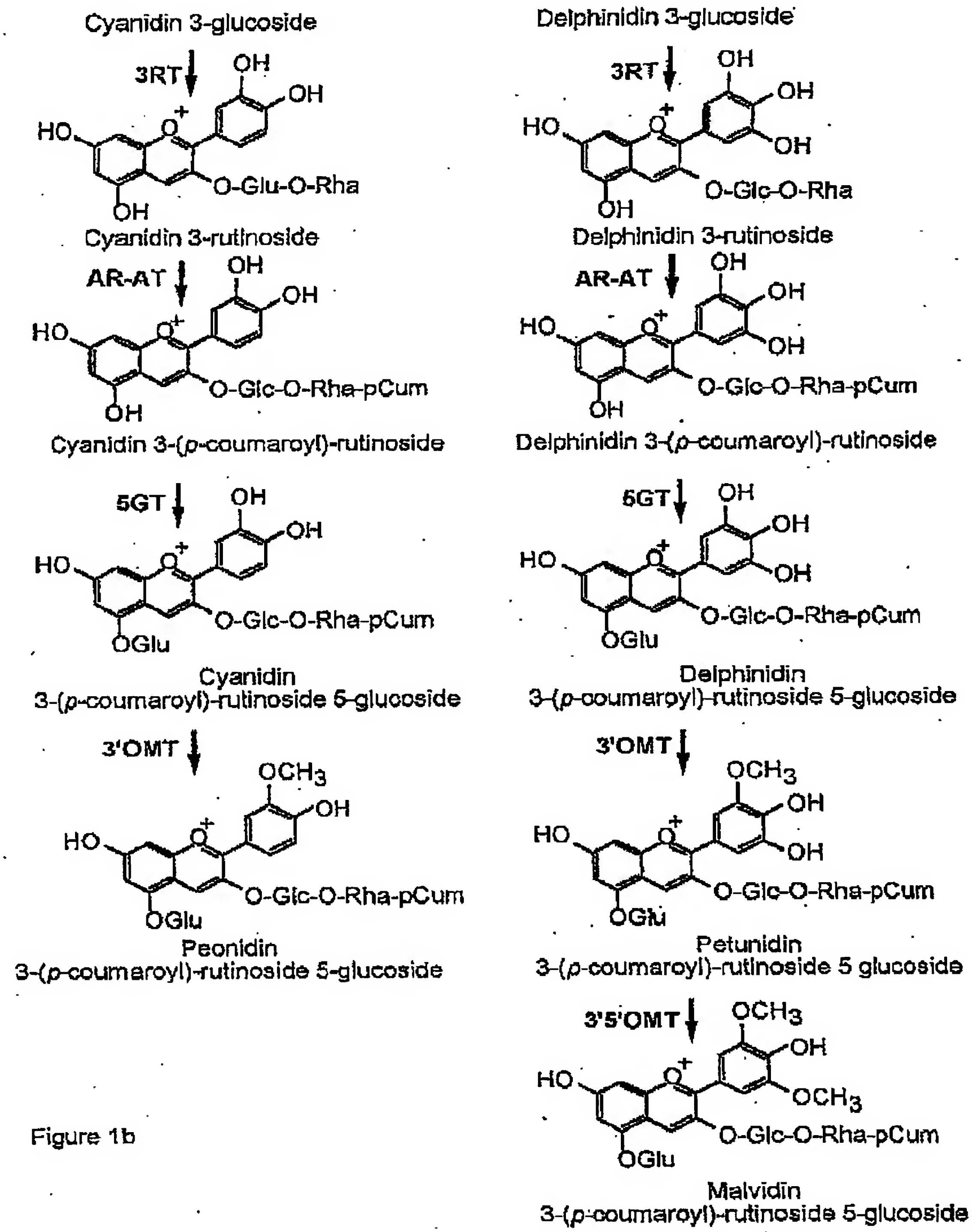
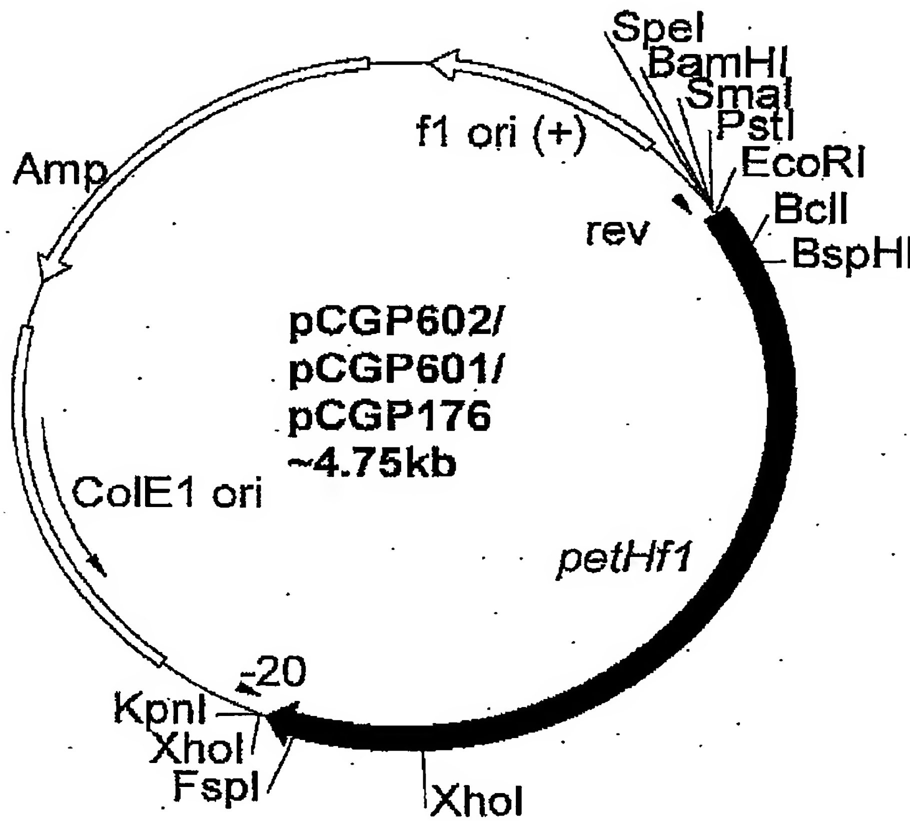

Cyanidin 3-glucoside
3RT
OH
OH
HO
O+
O-Glu-O-Rha
OH
Cyanidin 3-rutinoside
AR-AT
O-Glc-O-Rha-pCum
Cyanidin 3-(p-coumaroyl)-rutinoside
5GT
OGlu
Cyanidin
3-(p-coumaroyl)-rutinoside 5-glucoside
3'OMT
OCH3
Peonidin
3-(p-coumaroyl)-rutinoside 5-glucoside
Delphinidin 3-glucoside
O-Glc-O-Rha
Delphinidin 3-rutinoside
Delphinidin 3-(p-coumaroyl)-rutinoside
Delphinidin
3-(p-coumaroyl)-rutinoside 5-glucoside
Petunidin
3-(p-coumaroyl)-rutinoside 5 glucoside
3'5'OMT
Malvidin
3-(p-coumaroyl)-rutinoside 5-glucoside

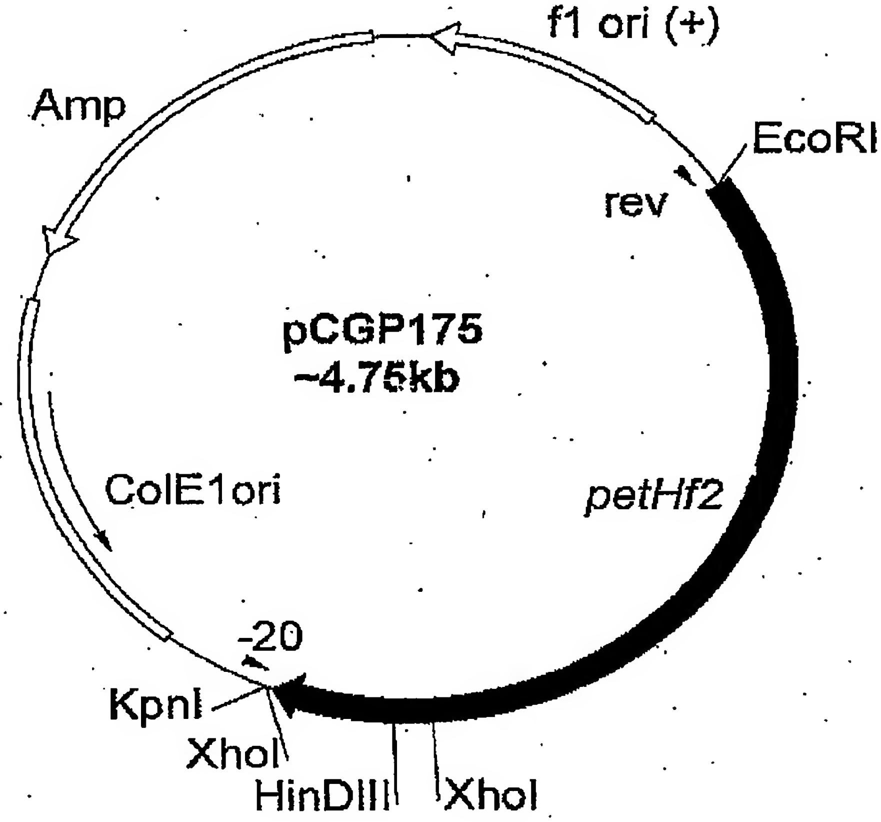
Figure 1b



Replicon: pBluescript SK (+) vector 2.95kb

Insert: ~1.8kb petunia F3'5'H *petHf1* cDNA homologs from *P. hybrida* cv. OGB

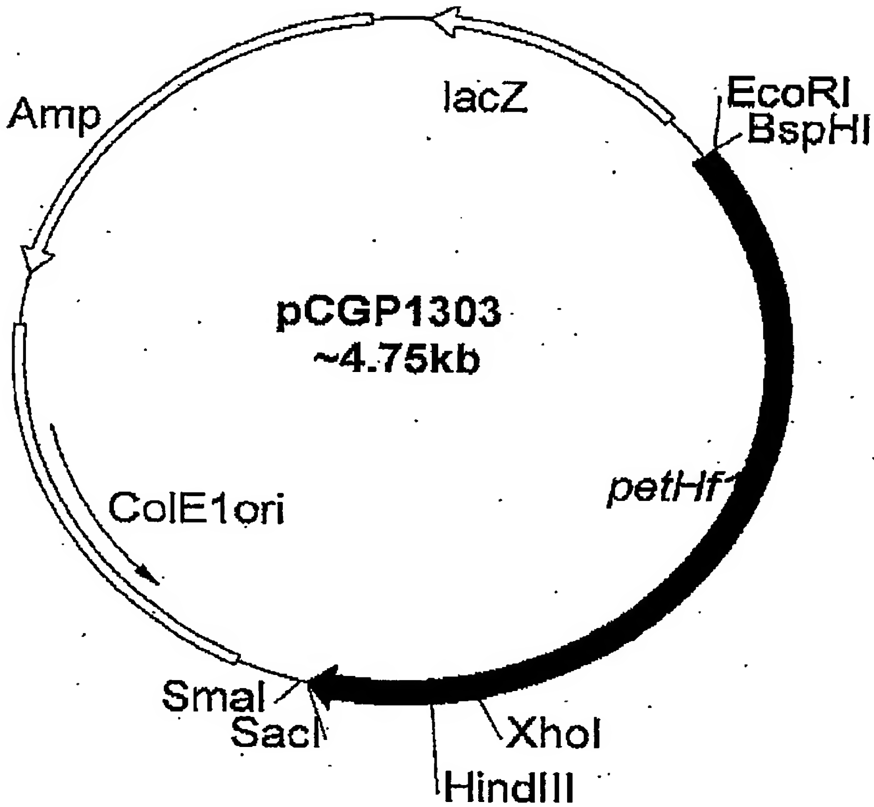
Figure 2



Replicon: pBluescript SK (+) vector 2.95kb

Insert: ~1.8kb petunia F3'5'H *petHf2* cDNA from *P. hybrida* cv. OGB

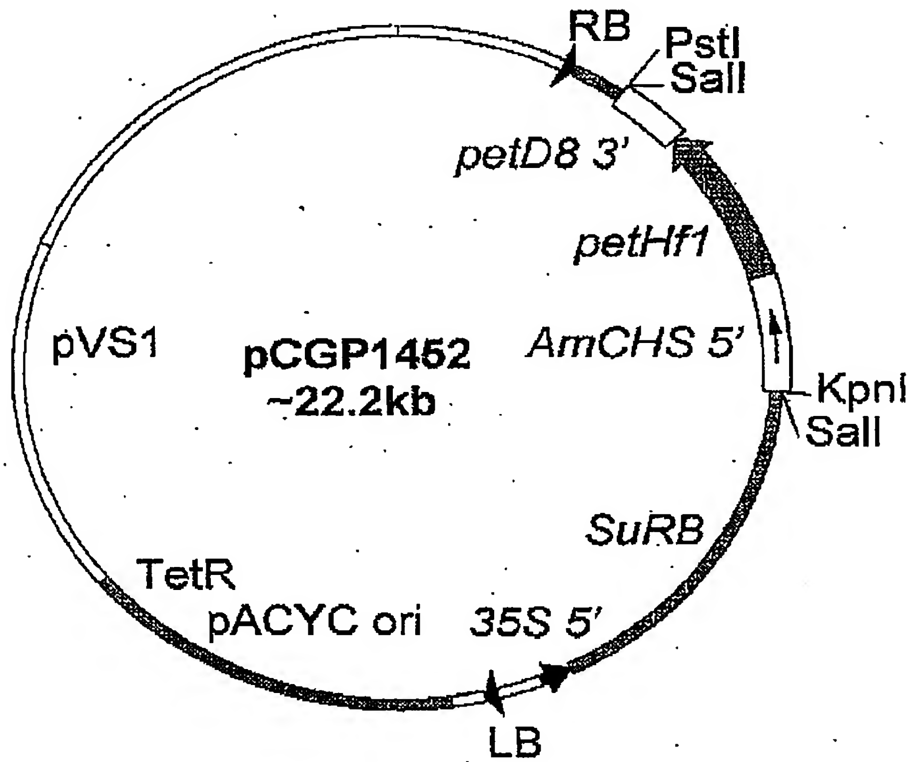
Figure 3



Replicon: ~2.7kb EcoRI (blunted) pUC19 vector

Insert: ~1.6kb BspHI (blunted)/FspI fragment containing petunia F3'5'H *petHf1* cDNA from pCGP601

Figure 4



Replicon: ~18.7kb SmaI pWTT2132 vector

Insert: ~3.5kb PstI (blunted) fragment containing *AmCHS 5': petHf1: petD8 3'* gene from pCGP485

Figure 5

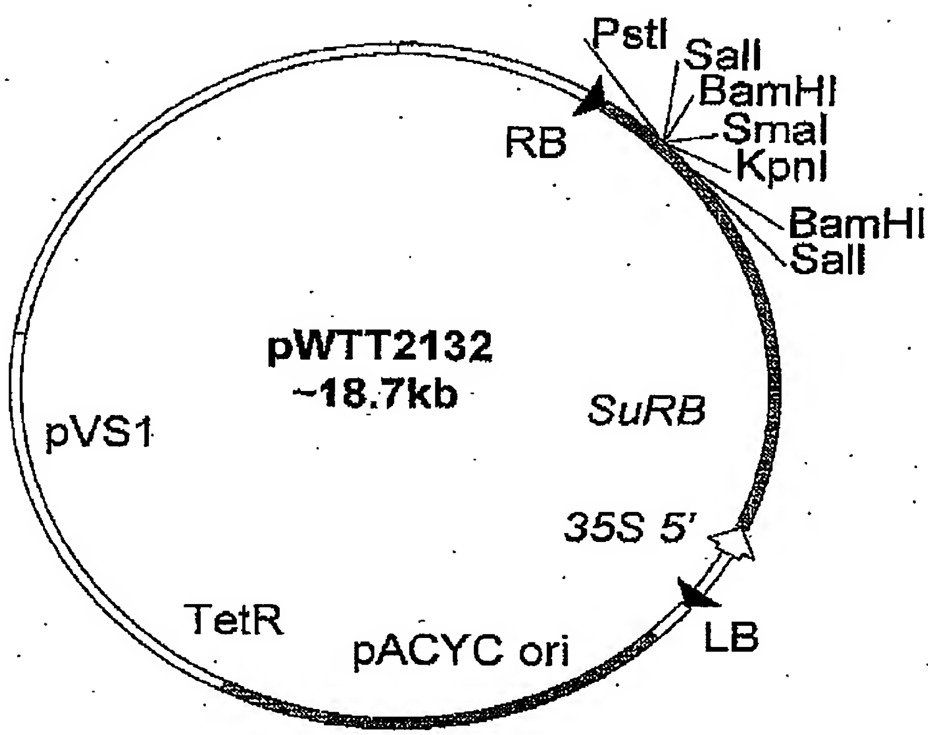
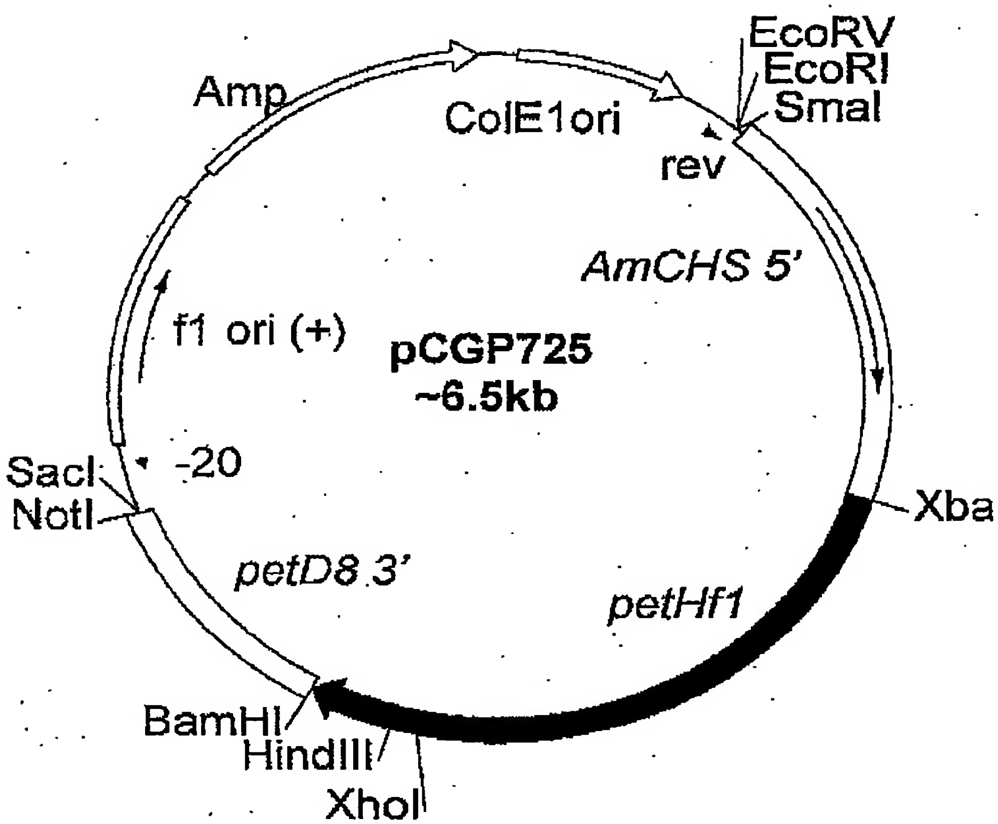

PstI
SalI
BamHI
SmaI
KpnI
BamHI
SalI
RB
pWTT2132
~18.7kb
SuRB
pVS1
35S 5'
TetR
pACYC ori
LB

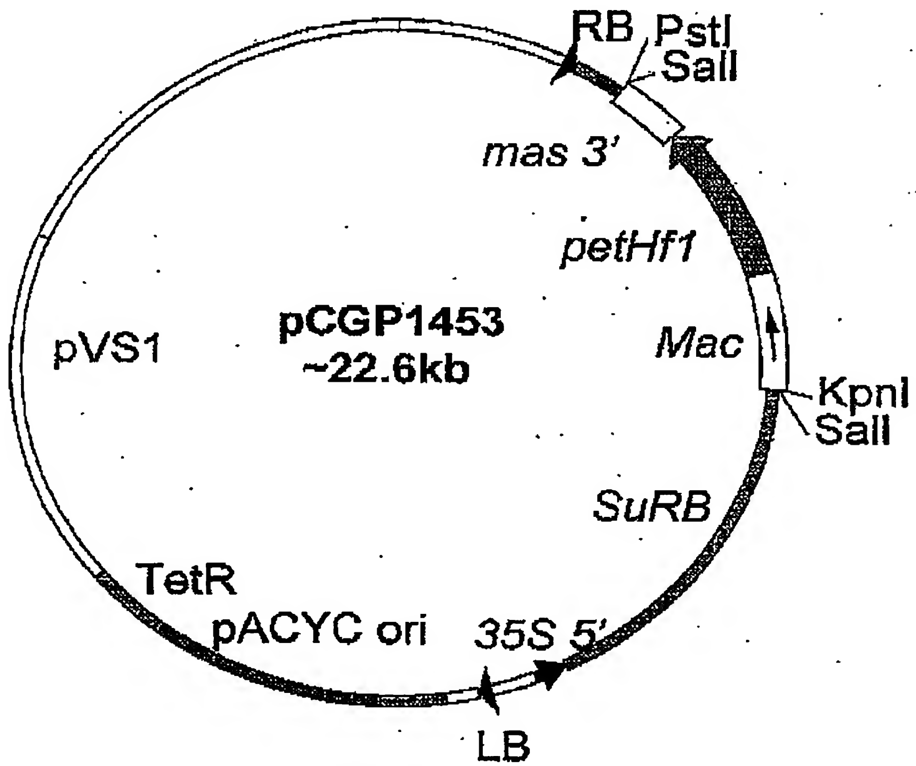
Figure 6



Replicon: 2.95kb (BamHI/XbaI) blunted vector fragment of pBluescript II KS (+).

Insert: ~3.5kb PstI (blunted) fragment containing *AmCHS 5': petHf1: petD8 3'* gene from pCGP483

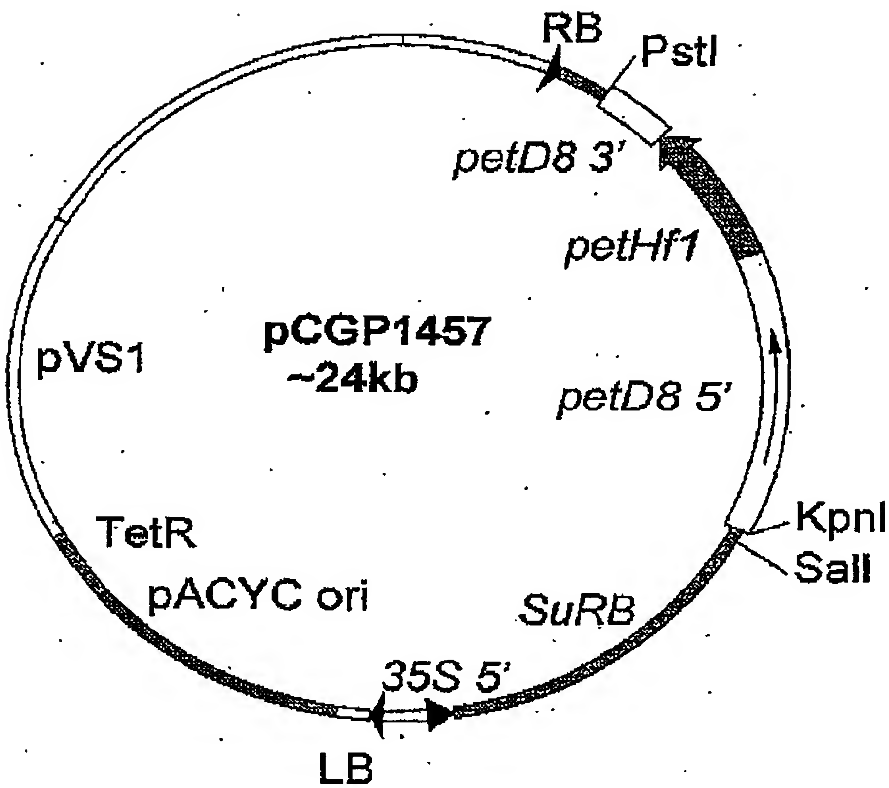
Figure 7



Replicon: ~18.7kb SmaI pWTT2132 vector

Insert: ~3.9kb PstI (blunted) fragment containing *Mac: petHf1: mas 3'* gene from pCGP628

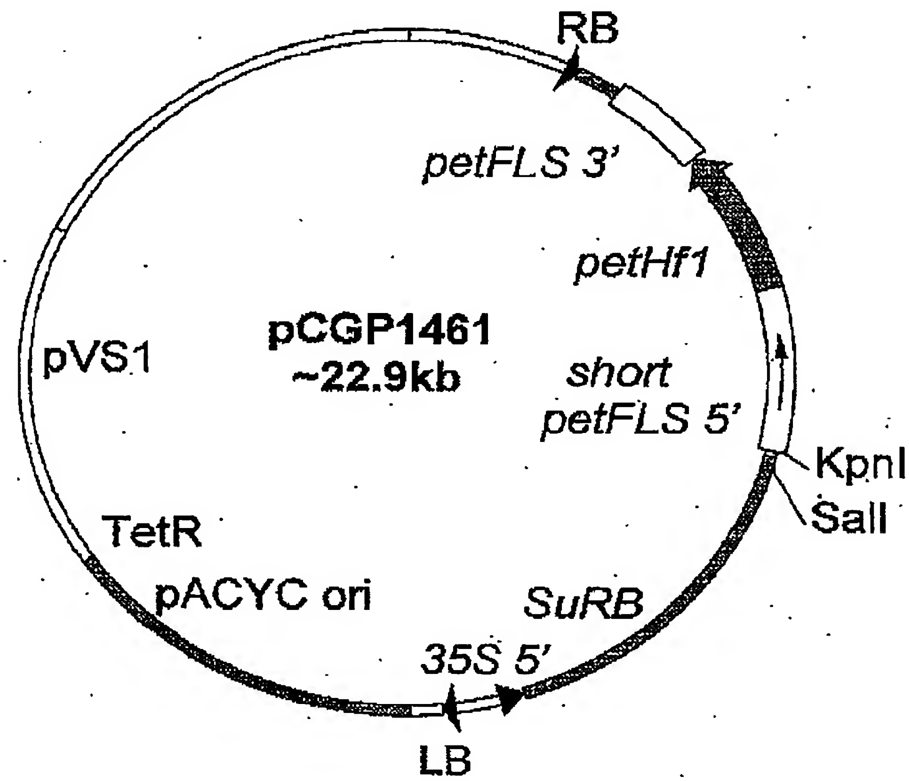
Figure 8



Replicon: ~18.7kb SmaI/PstI pWTT2132 vector

Insert: ~5.3kb XbaI (blunted)/PstI fragment containing *petD8 5': petHf1: petD8 3'* gene from pCGP1107

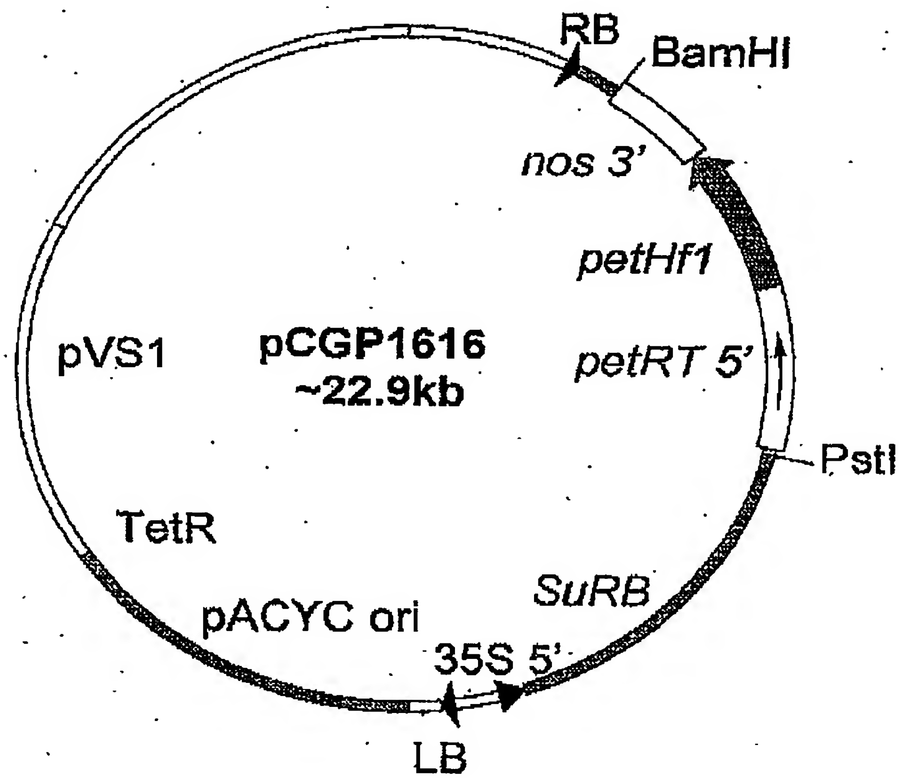
Figure 9



Replicon: ~18.7kb PstI (blunted)/KpnI pWTT2132 vector

Insert: ~4.35kb SacI (blunted)/KpnI fragment containing *shortFLS 5': petHf1: petFLS 3'* gene from pCGP497

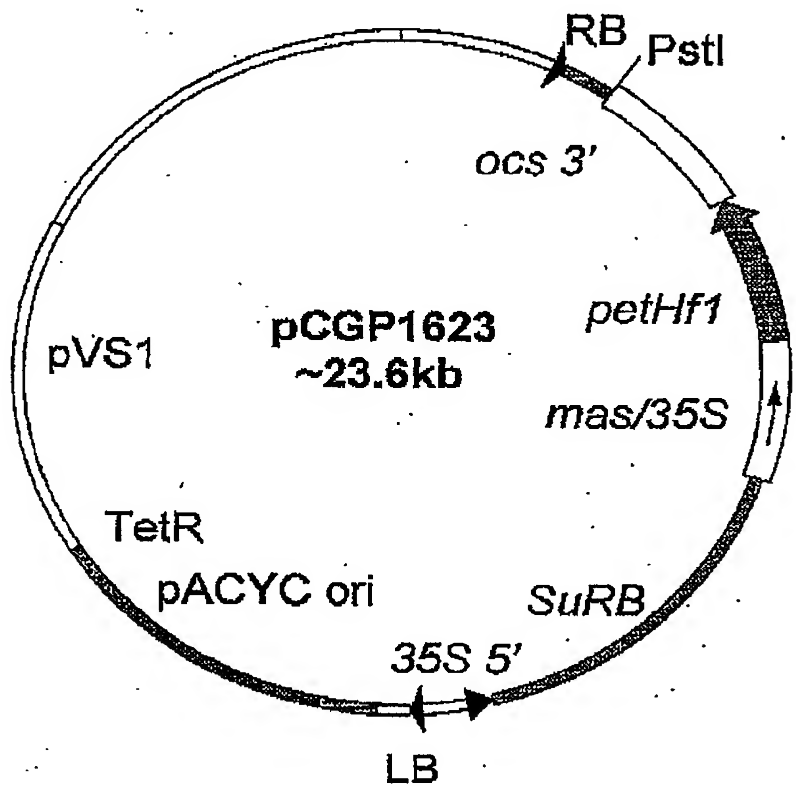
Figure 10



Replicon: ~18.7kb PstI/BamHI pWTT2132 vector

Insert: ~3kb PstI/BamHI fragment containing *petRT5': petHf1: nos 3'* gene from pCGP846

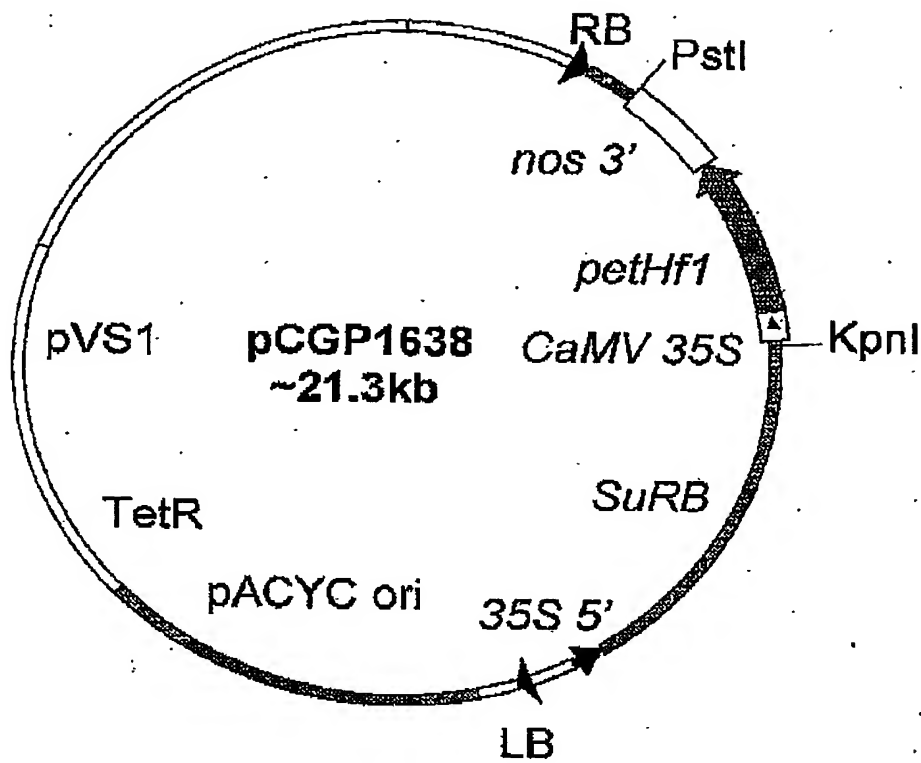
Figure 11



Replicon: ~18.7kb SalI pWTT2132 vector

Insert: ~4.9kb XhoI fragment containing
mas/35S: petHf1: ocs 3' gene from pCGP1619

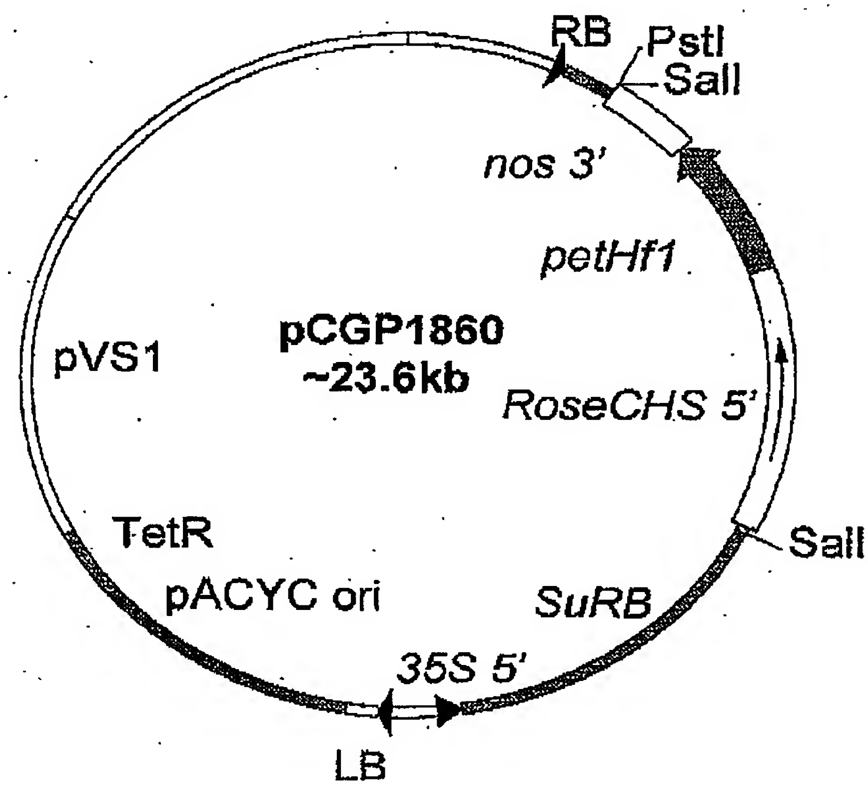
Figure 12



Replicon: ~18.7kb SmaI pWTT2132 vector

Insert: ~2.6kb (PstI/EcoRI) blunted fragment containing *CaMV 35S: petHf1: ocs 3'* gene from pCGP1636

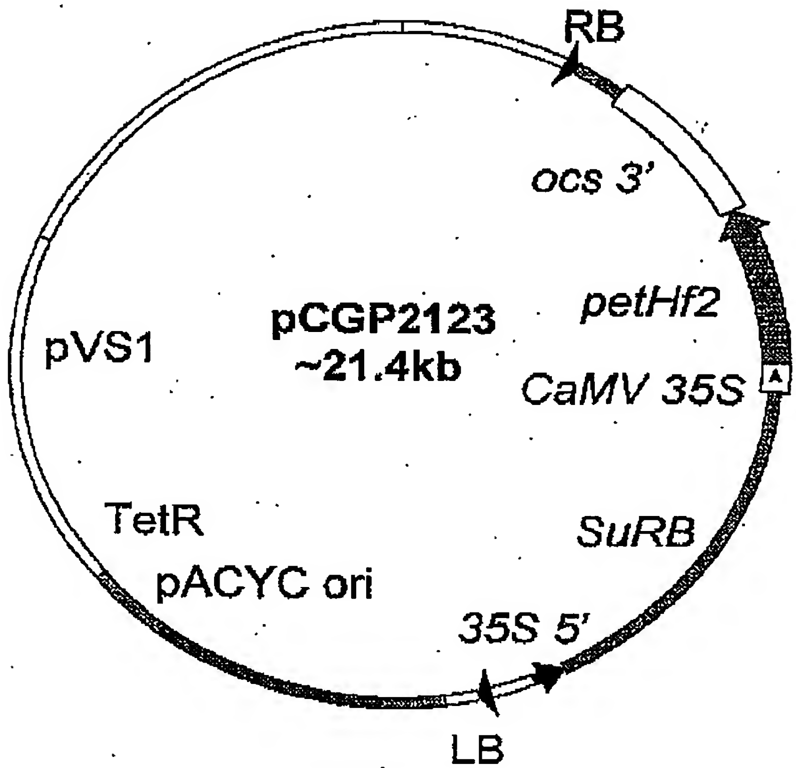
Figure 13



Replicon: ~18.7kb BamHI pWTT2132 vector

Insert: ~4.9kb BglII fragment from containing *RoseCHS 5';petHf1: nos 3'* gene from pCGP200

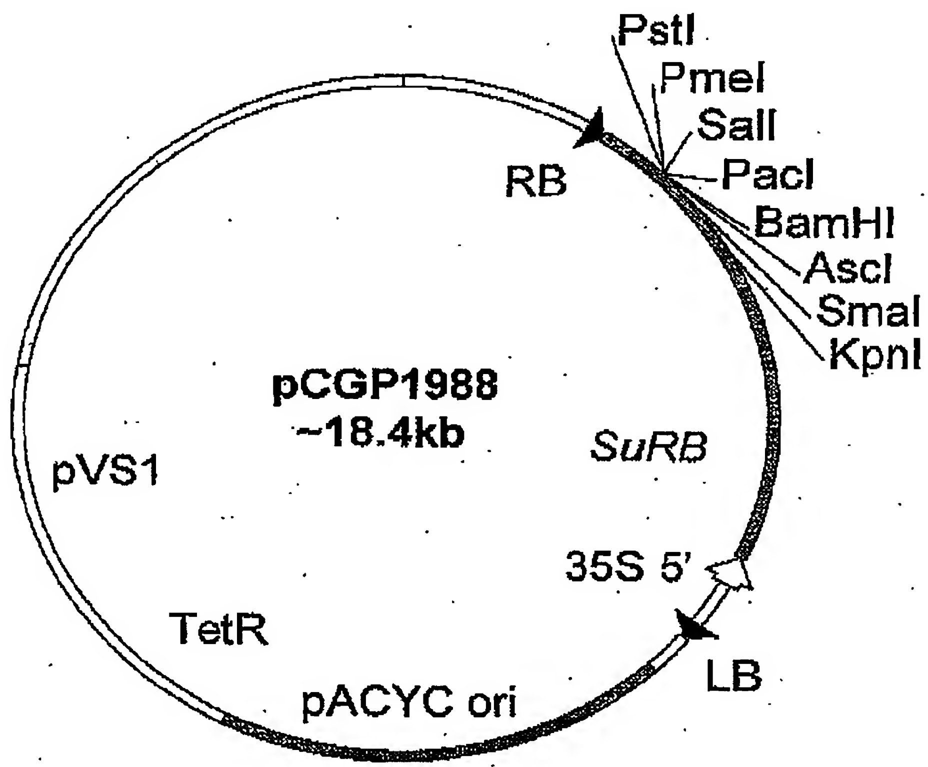
Figure 14



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.7kb (Asp718/XbaI) blunted fragment containing *CaMV 35S: petHf2: ocs 3'* gene from pCGP2109

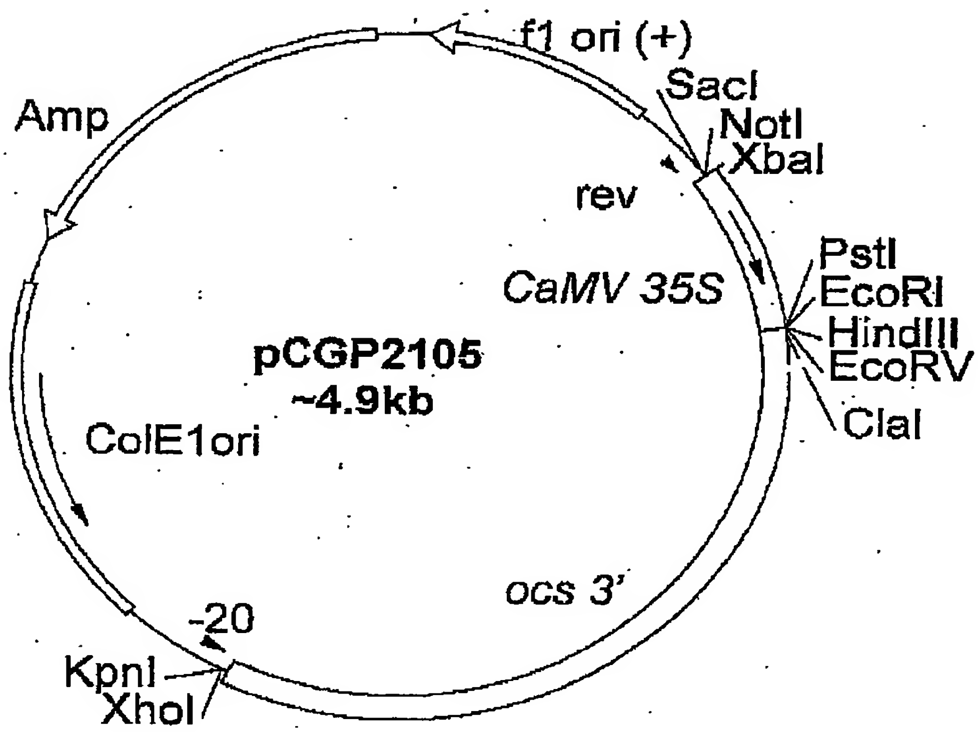
Figure 15



Replicon: ~18.4kb SalI (blunted)/PstI vector fragment from pWTT2132

Insert: ~66bp EcoRI (blunted)/ PstI fragment containing multi-cloning site from pNEB193

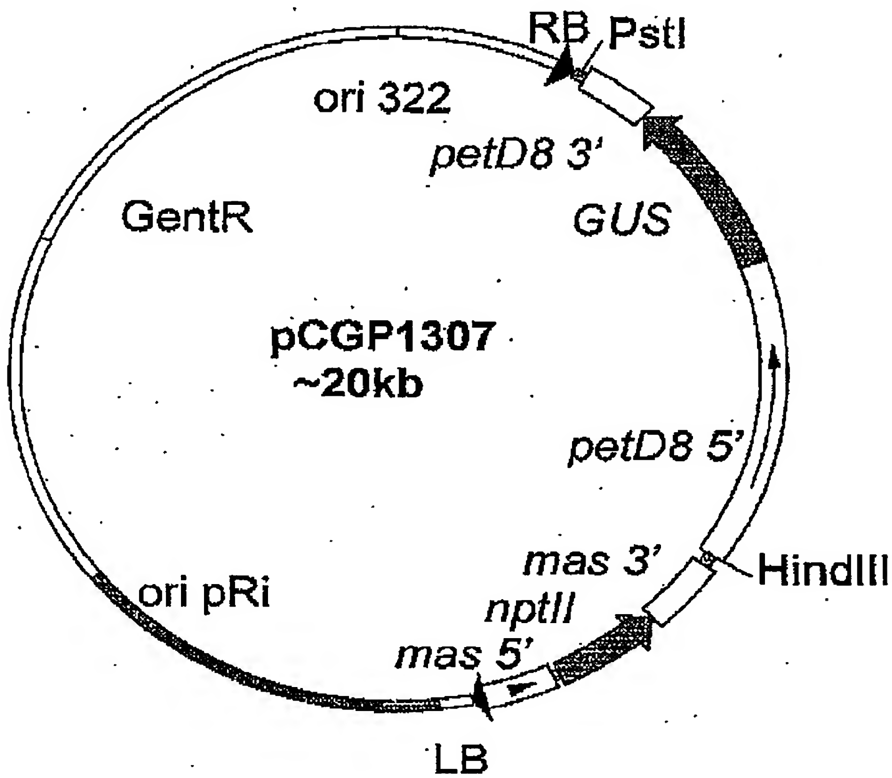
Figure 16



Replicon: ~3.3 kb HincII/XhoI vector fragment from pCGP2000 (containing *CaMV 35S* promoter fragment in pBluescript SK)

Insert: ~1.6kb EcoRI (blunted)/ XhoI *ocs 3'* fragment from pKIWI101

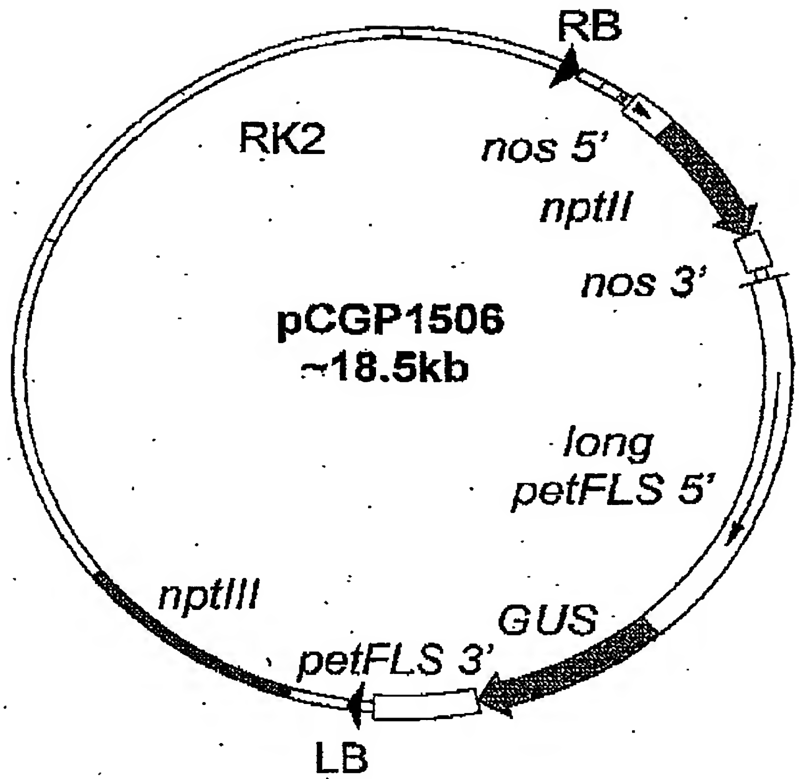
Figure 17



Replicon: ~15kb HindIII/PstI pCGN1548 vector

Insert: ~5.3kb HindIII/PstI fragment containing *petD8 5': GUS: petD8 3'* gene from pCGP1106

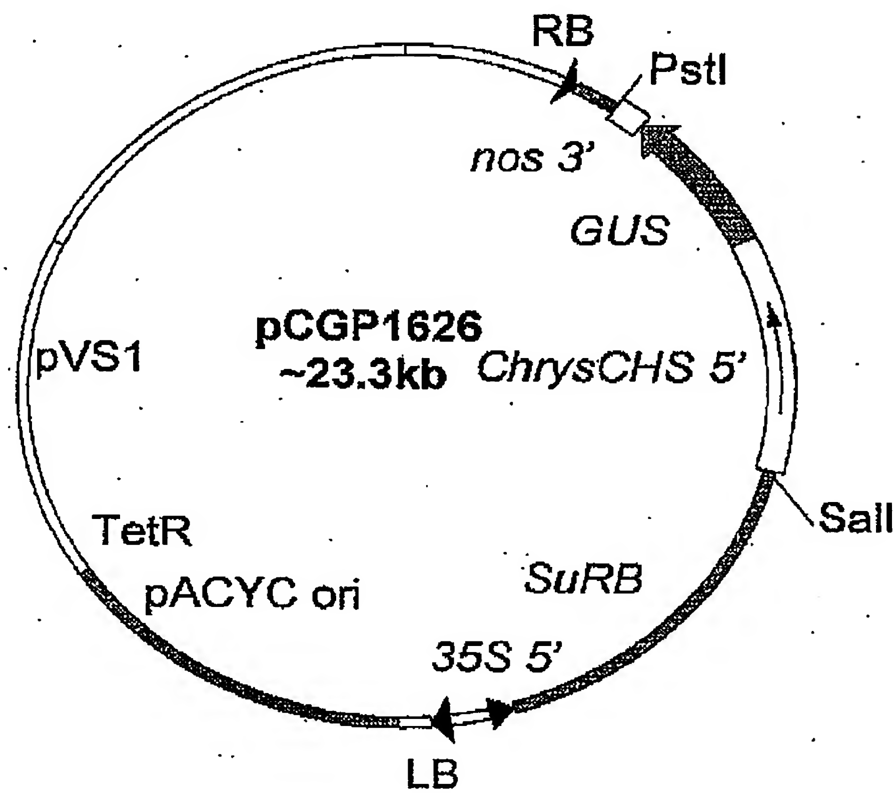
Figure 18



Replicon:~11.8kb BamHI (GA-filled)/SacI pBIN19 vector

Insert: ~6.7kb XhoI (TC-filled)/SacI fragment containing *longpetFLS 5': GUS: petFLS 3'* gene from pCGP496

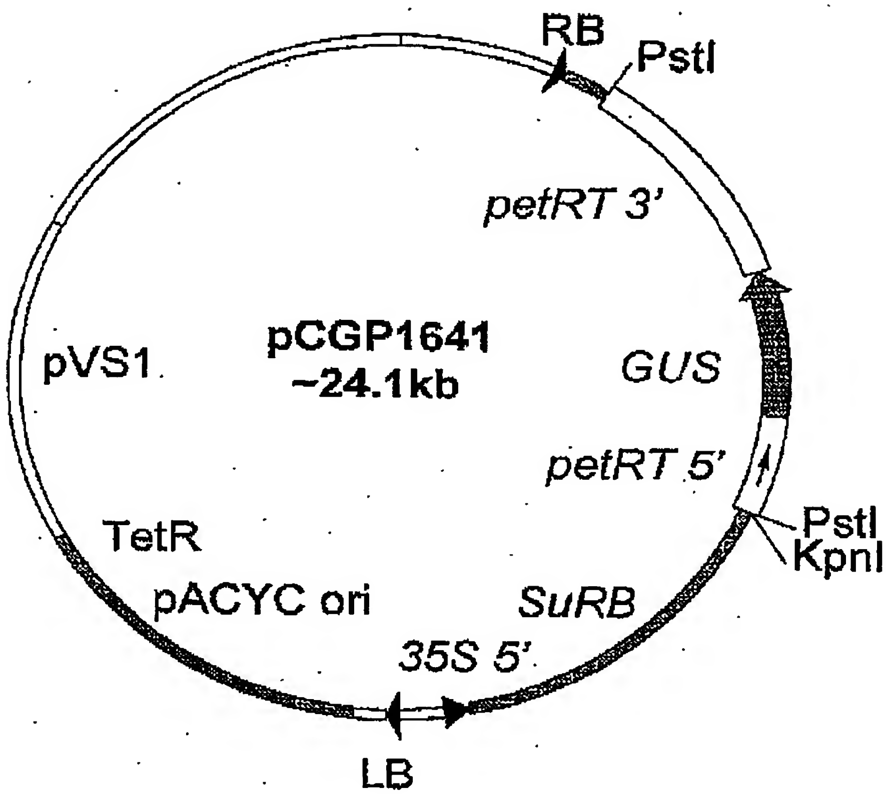
Figure 19



Replicon: ~18.7kb PstI/BamHI pWTT2132 vector

Insert: ~4.6kb PstI/BglII fragment containing *ChrysCHS 5': GUS: nos 3'* gene from pCGP1622

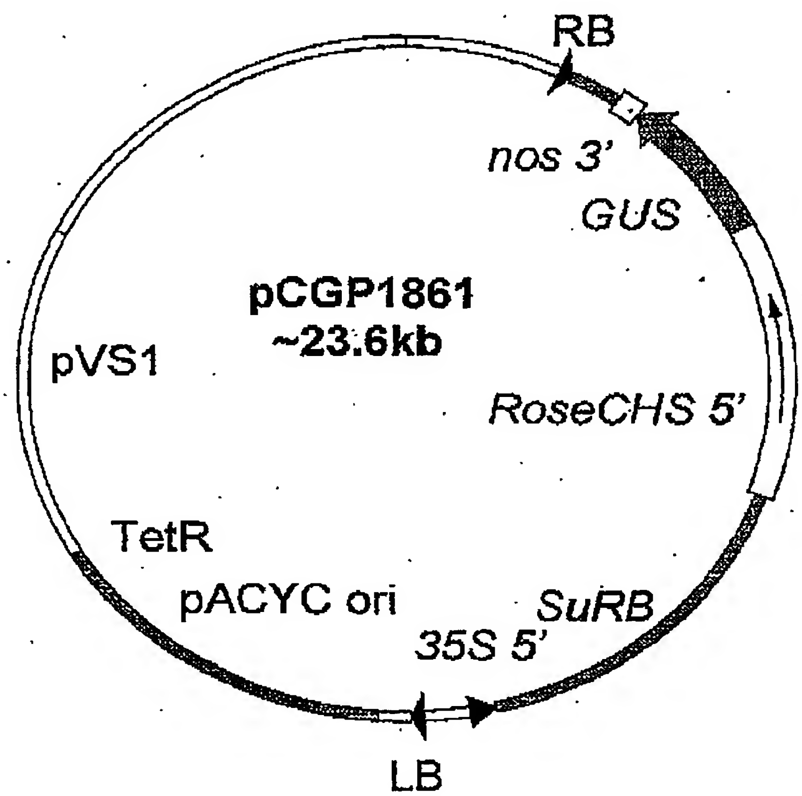
Figure 20



Replicon: ~18.7kb PstI pWTT2132 vector

Insert: ~5.4kb PstI fragment containing *petRT 5': GUS: petRT 3' gene* from pCGP1628

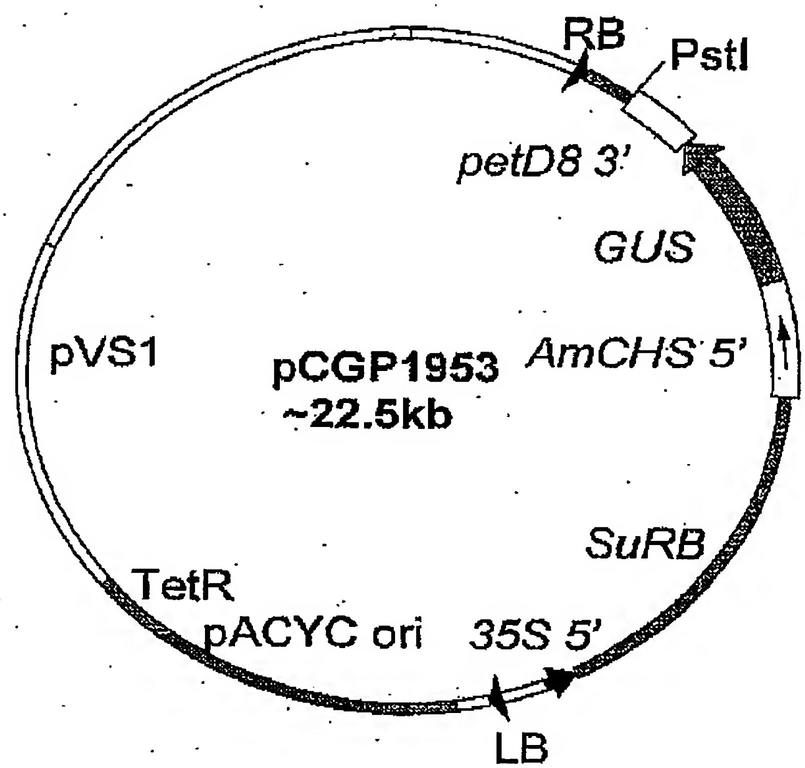
Figure 21



Replicon: ~18.7kb BamHI pWTT2132 vector

Insert: ~5kb BglII fragment containing *RoseCHS 5': GUS: nos 3'* gene from pCGP197

Figure 22



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.8kb (EagI/PstI) blunted fragment containing *AmCHS 5': GUS: petD8 3'* gene from pCGP1952

Figure 23

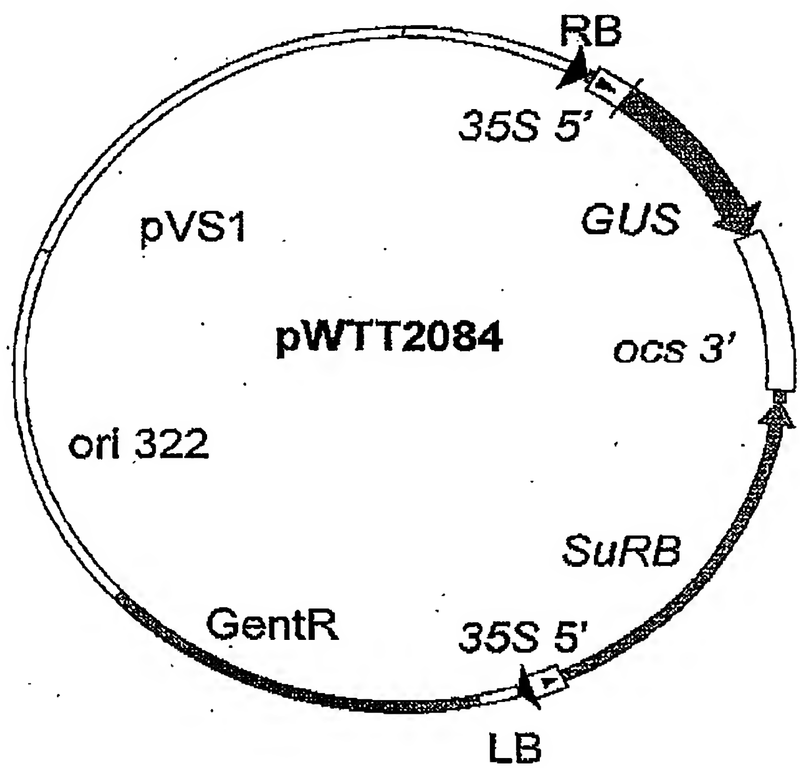
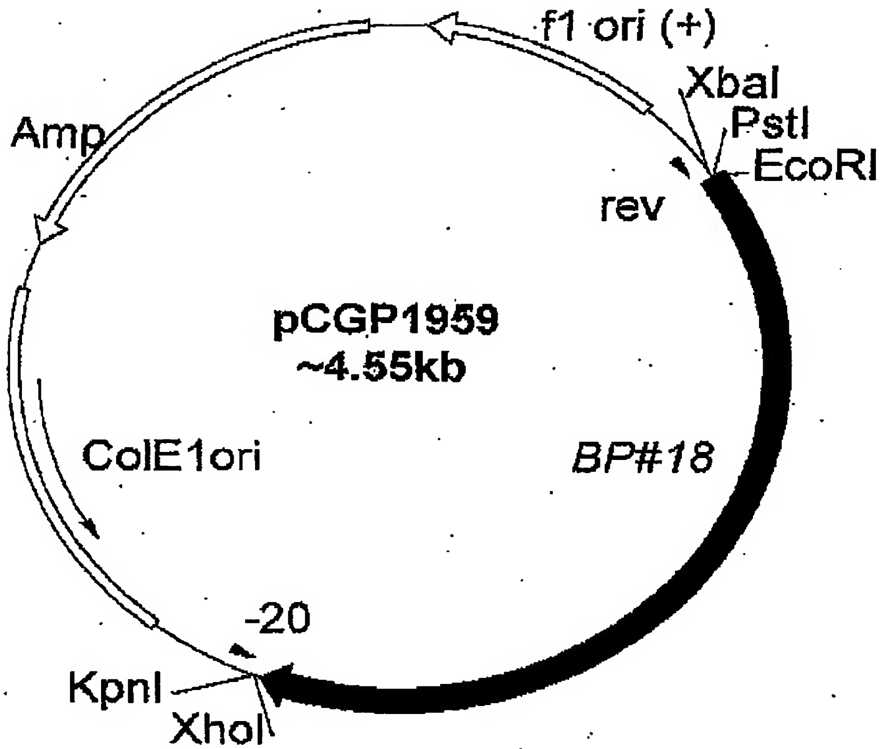


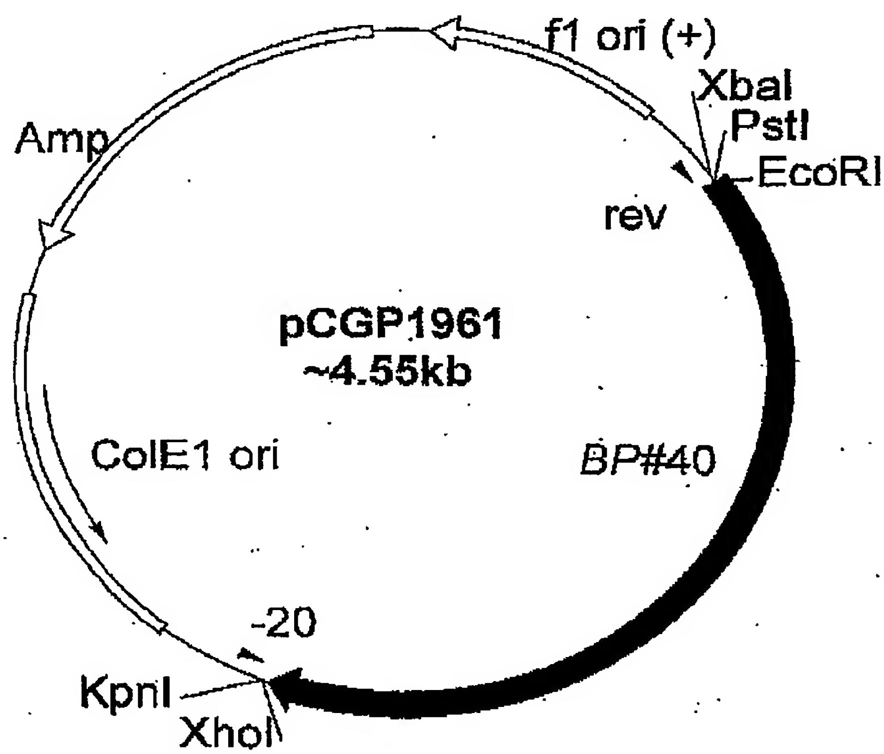
Figure 24



Replicon: pBluescript SK II (+) vector 2.95kb

Insert: ~1.6kb *pansy F3'5'H BP#18* cDNA from *Viola spp.* cv. Black Pansy

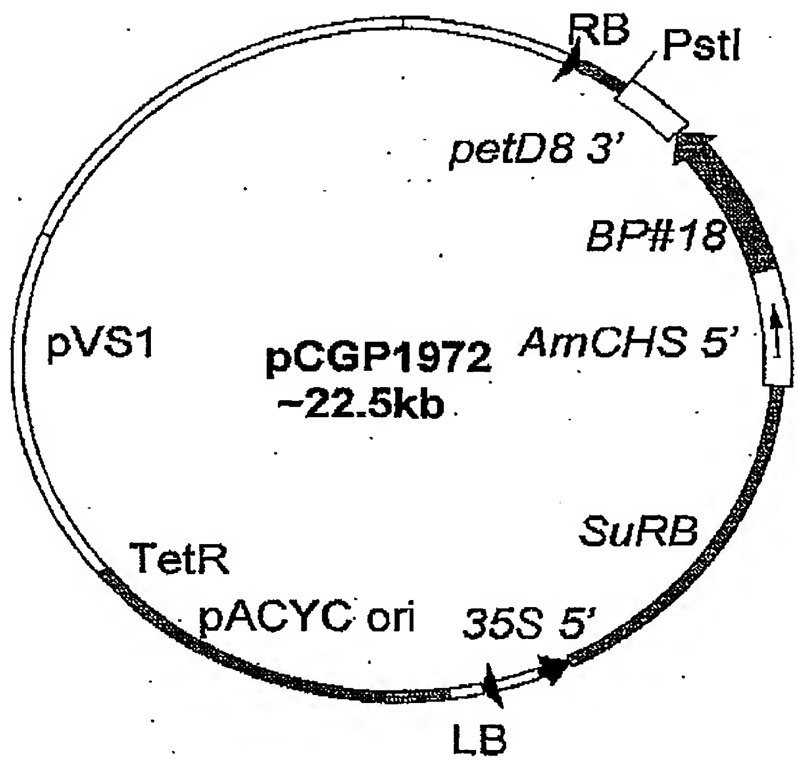
Figure 25



Replicon: pBluescript SK II (+) vector
2.95kb

Insert: ~1.6kb *pansy F3'5'H BP#40* cDNA
from *Viola spp.* cv. Black Pansy

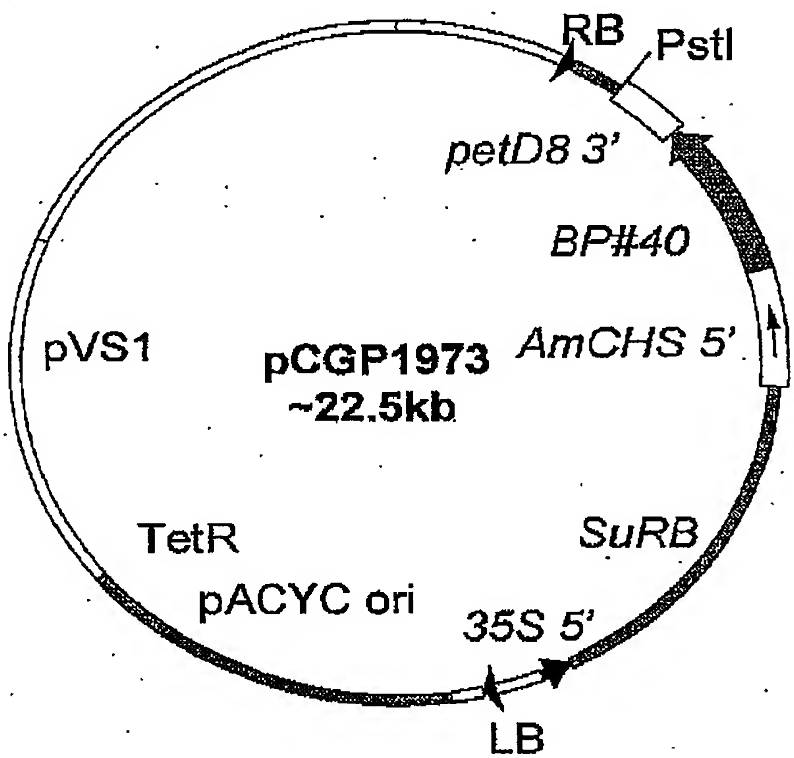
Figure 26



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.8kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': BP#18: petD8 3'* gene from pCGP1970

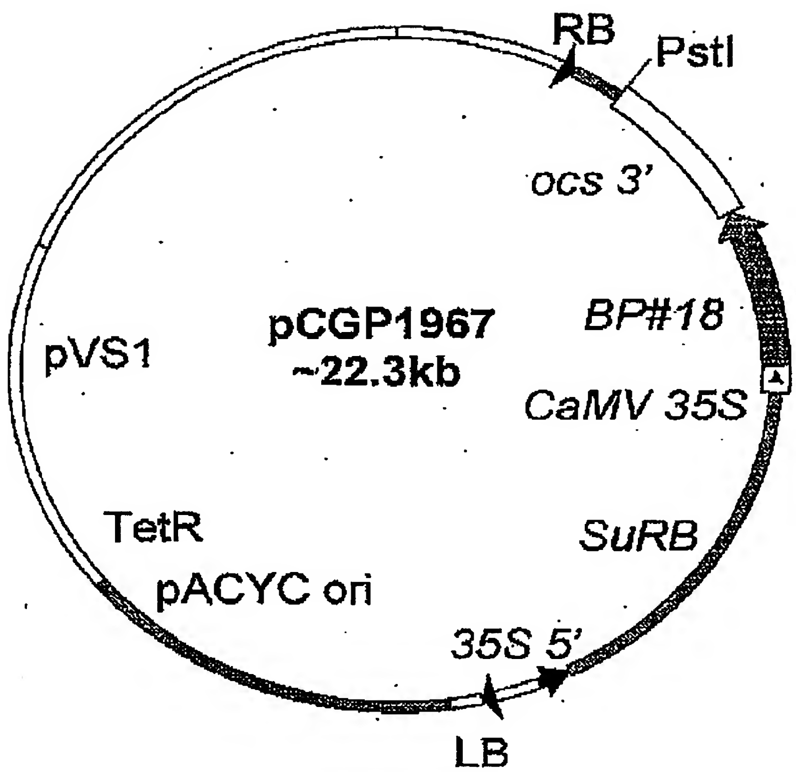
Figure 27



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.8kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': BP#40: petD8 3'* gene from pCGP1971

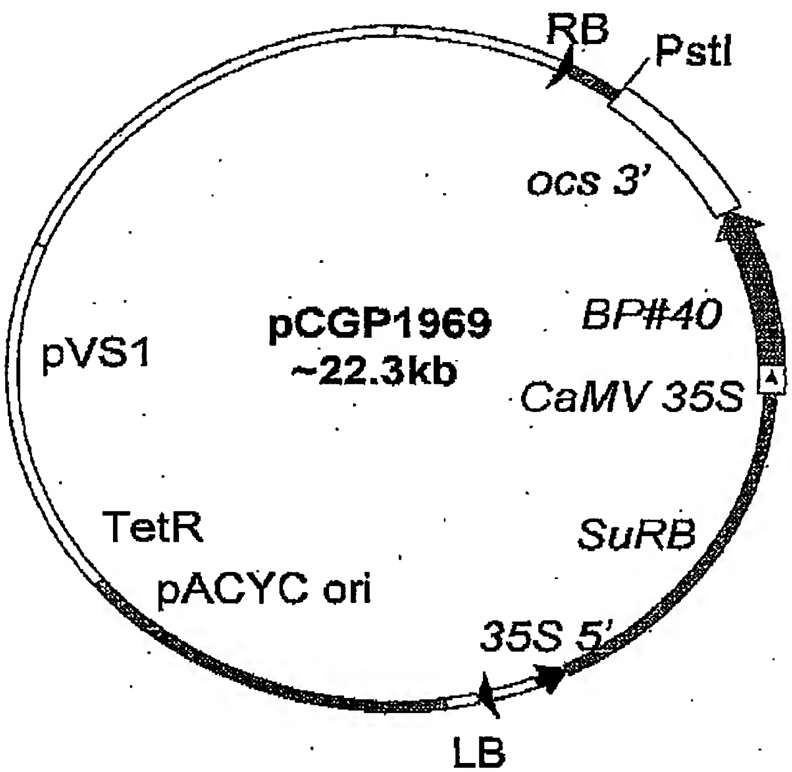
Figure 28



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.6kb (XhoI /XbaI) blunted fragment containing *CaMV 35S: BP#18: ocs 3'* gene from pCGP1965

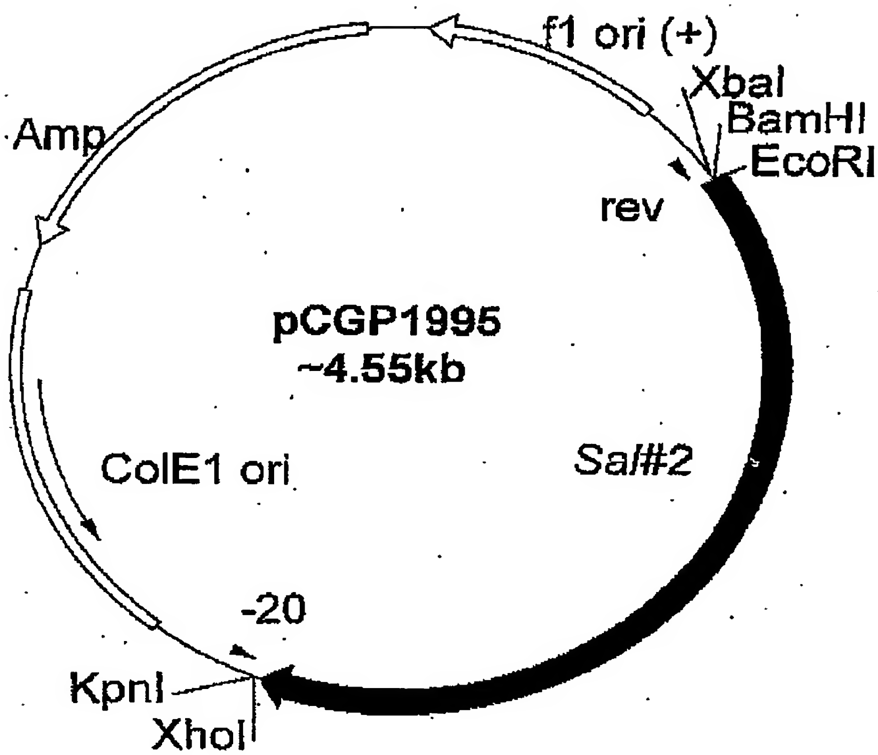
Figure 29



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.6kb (XhoI/XbaI) blunted fragment containing *CaMV 35S: BP#40: ocs 3'* gene from pCGP1966

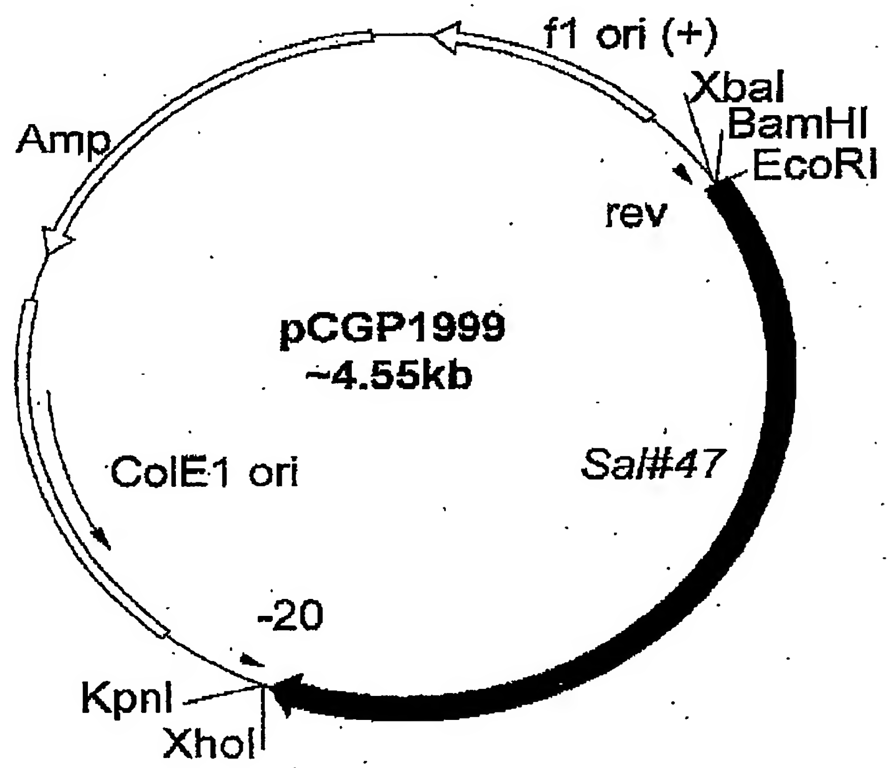
Figure 30



Replicon: pBluescript SK II (+) vector 2.95kb

Insert: ~1.6kb *F3'5'H Sal#2* cDNA from *Salvia spp.*

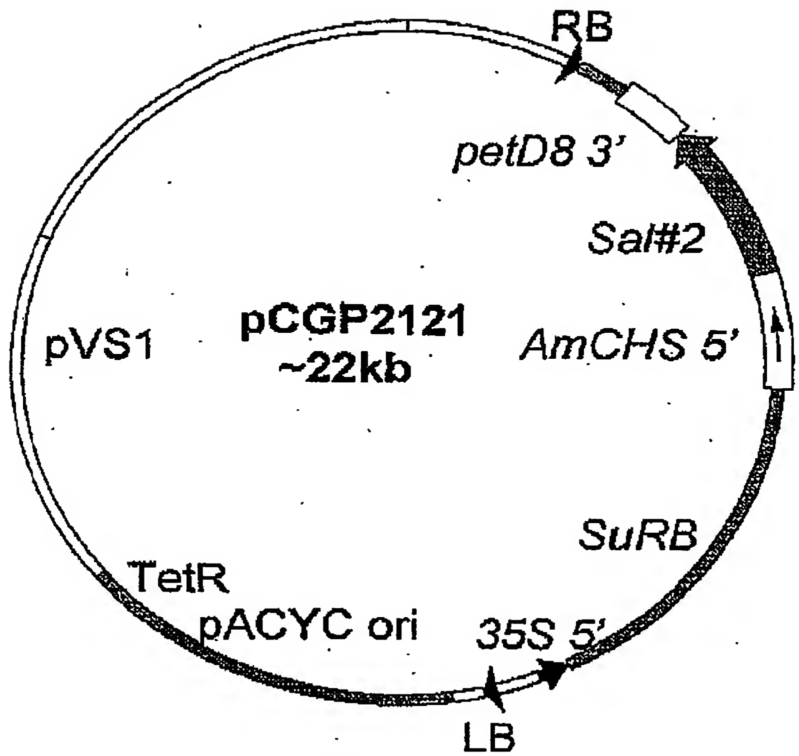
Figure 31



Replicon: pBluescript SK II (+) vector
2.95kb

Insert: ~1.6kb *F3'5'H Sal#47* cDNA from
Salvia spp.

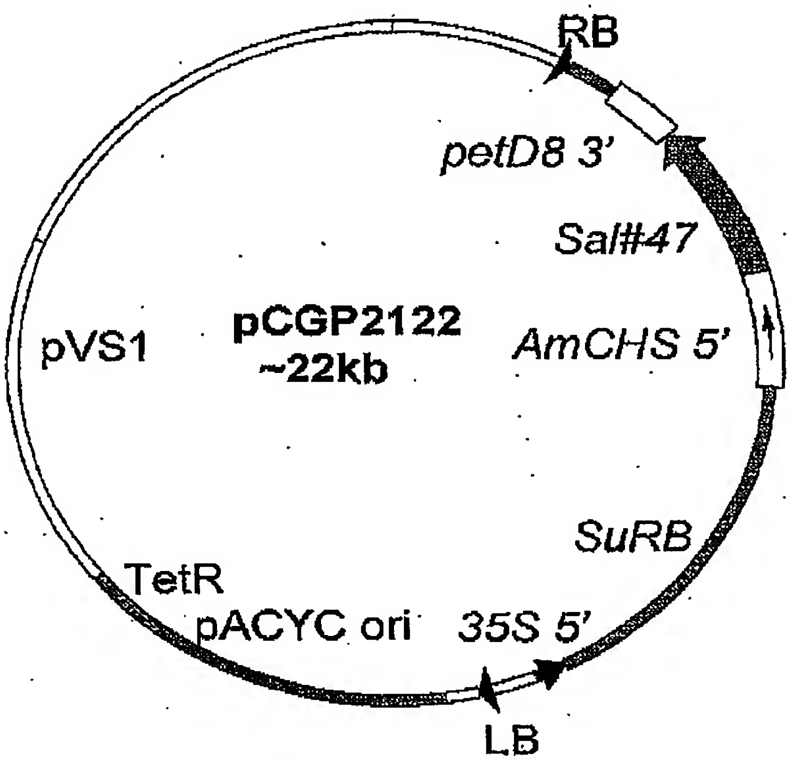
Figure 32



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': Sal#2: petD8 3'* gene from pCGP2116

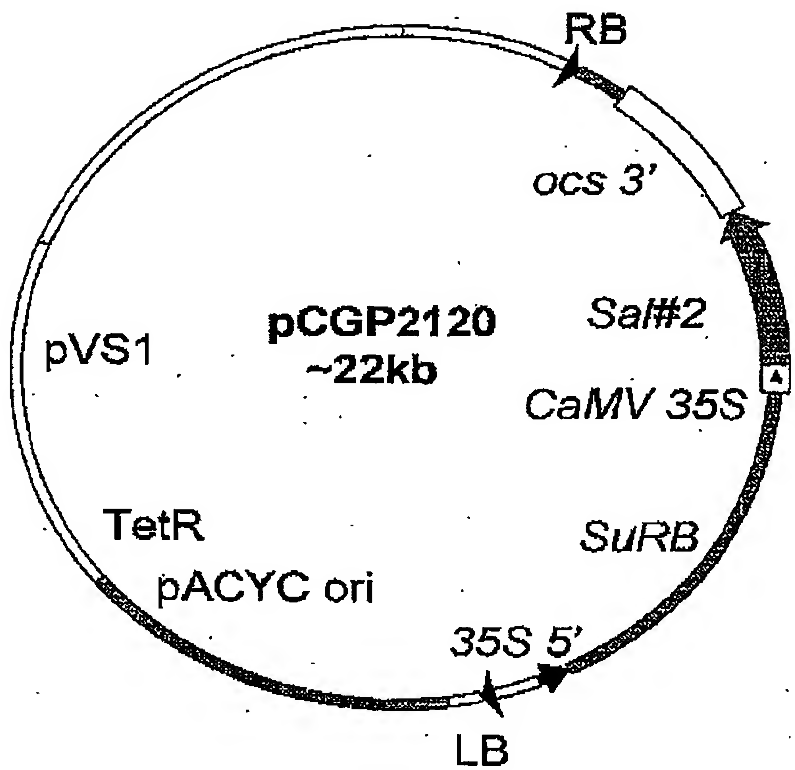
Figure 33



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5'*: *Sal#47*: *petD8 3'* gene from pCGP2117

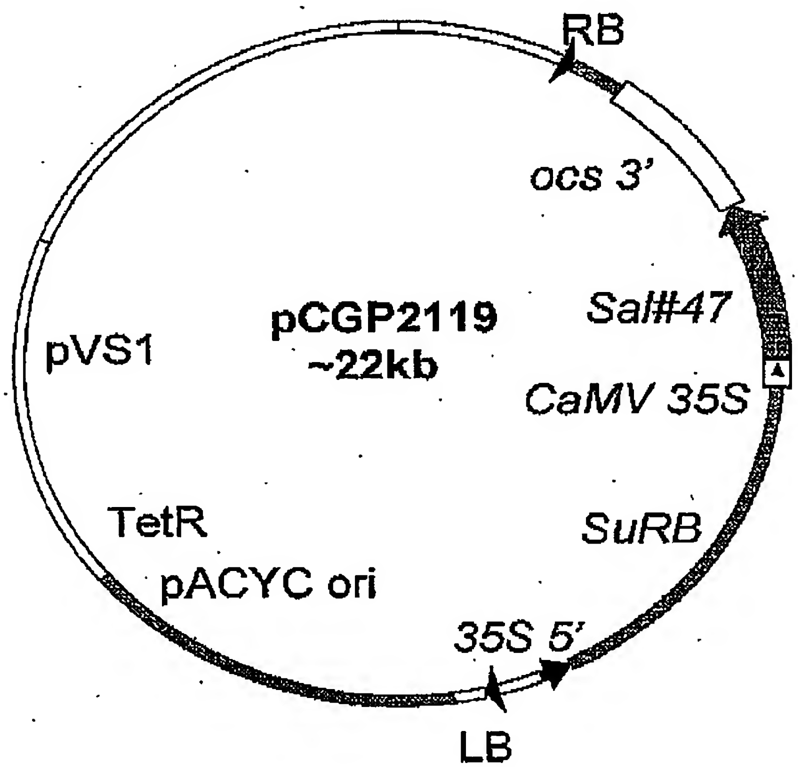
Figure 34



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb (XhoI/XbaI) blunted fragment containing *CaMV 35S: Sal#2: ocs 3'* gene from pCGP2112

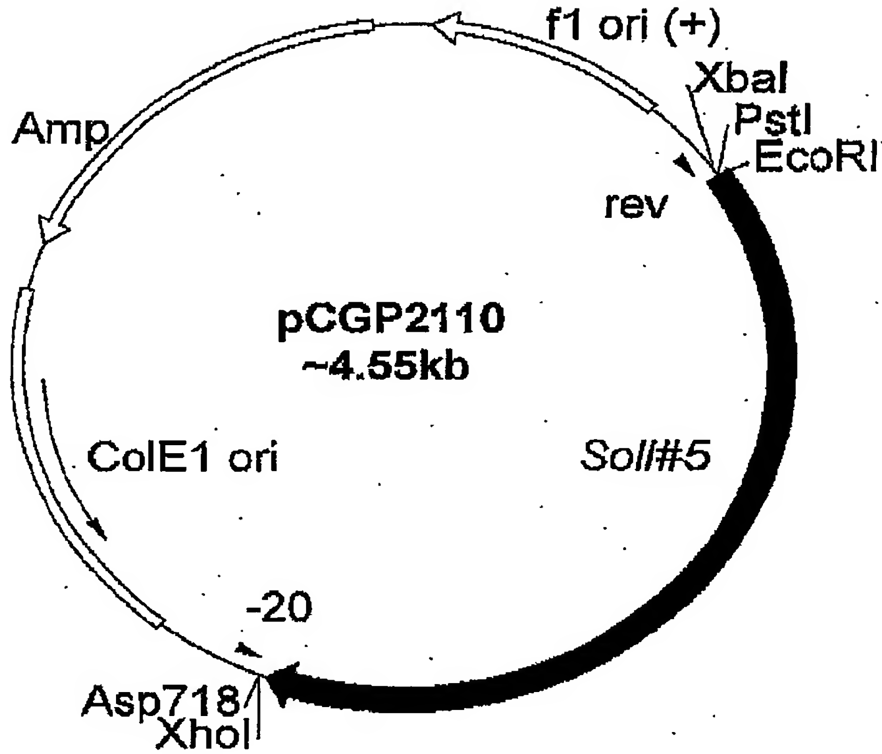
Figure 35



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb (XhoI/XbaI) blunted fragment containing *CaMV 35S: Sal#47: ocs 3'* gene from pCGP2111

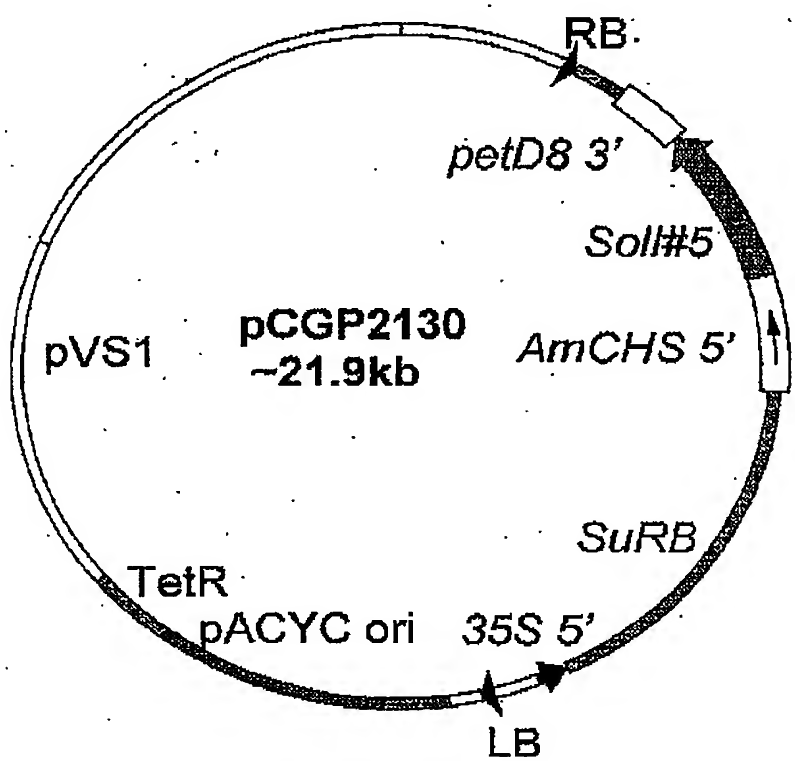
Figure 36



Replicon: pBluescript SK II (+) vector
2.95kb

Insert: ~1.7kb *F3'5'H SolI#5* cDNA
from *Sollya spp*.

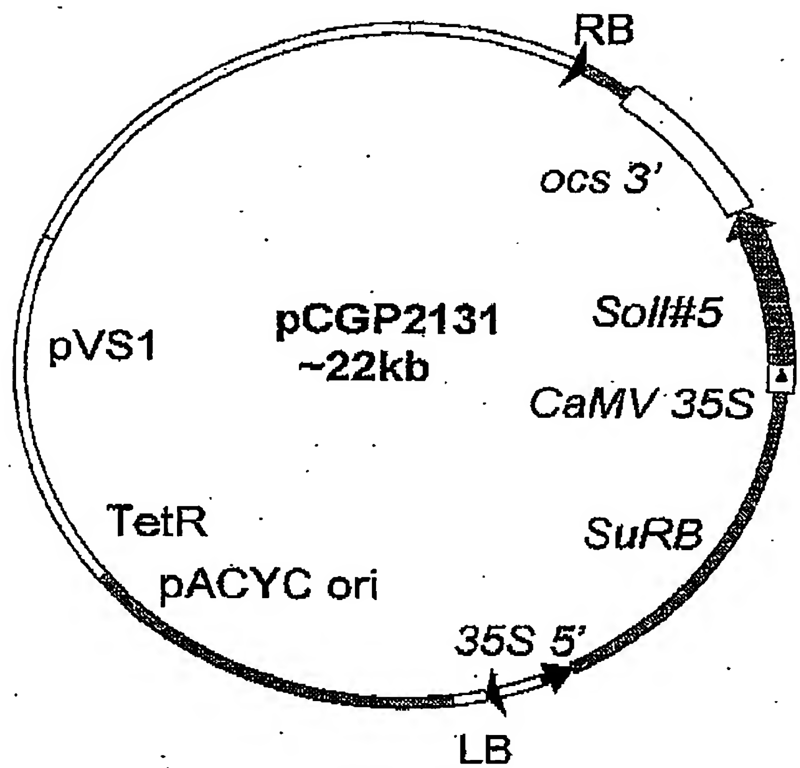
Figure 37



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.5kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': SolI#5: petD8 3'* gene from pCGP2128

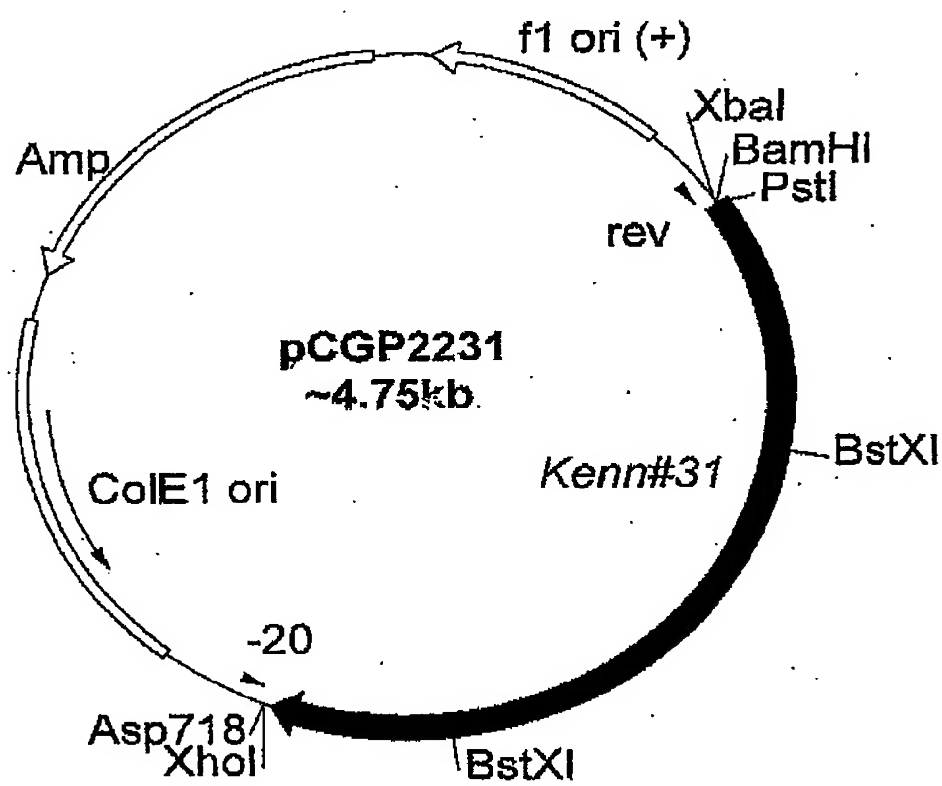
Figure 38



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb (Asp718/XbaI) blunted fragment containing *CaMV 35S: SolI#5: ocs 3'* gene from pCGP2129

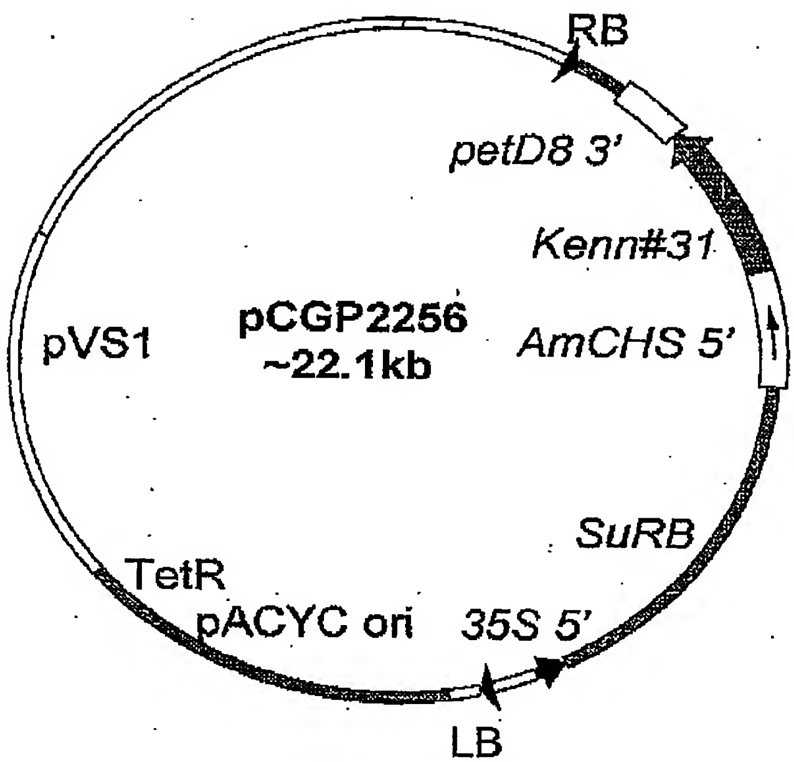
Figure 39



Replicon: pBluescript SK II (+) vector 2.95kb

Insert: ~1.8kb *F3'5'H Kenn#31* cDNA from *Kennedia spp.*

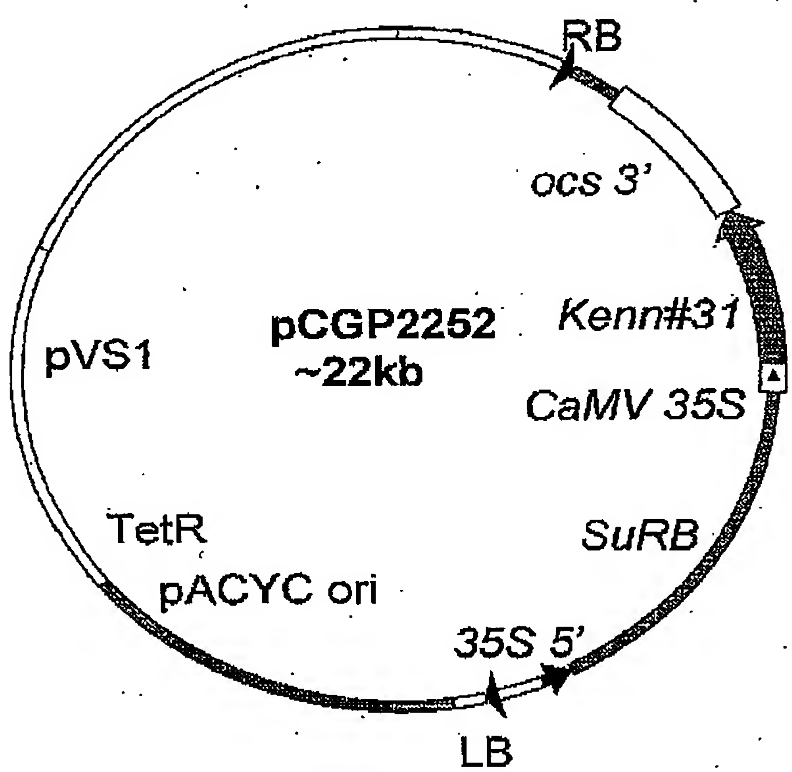
Figure 40



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.7kb (NotI/ EcoRI) blunted fragment containing *AmCHS 5': Kenn#31: petD8 3'* gene from pCGP2242

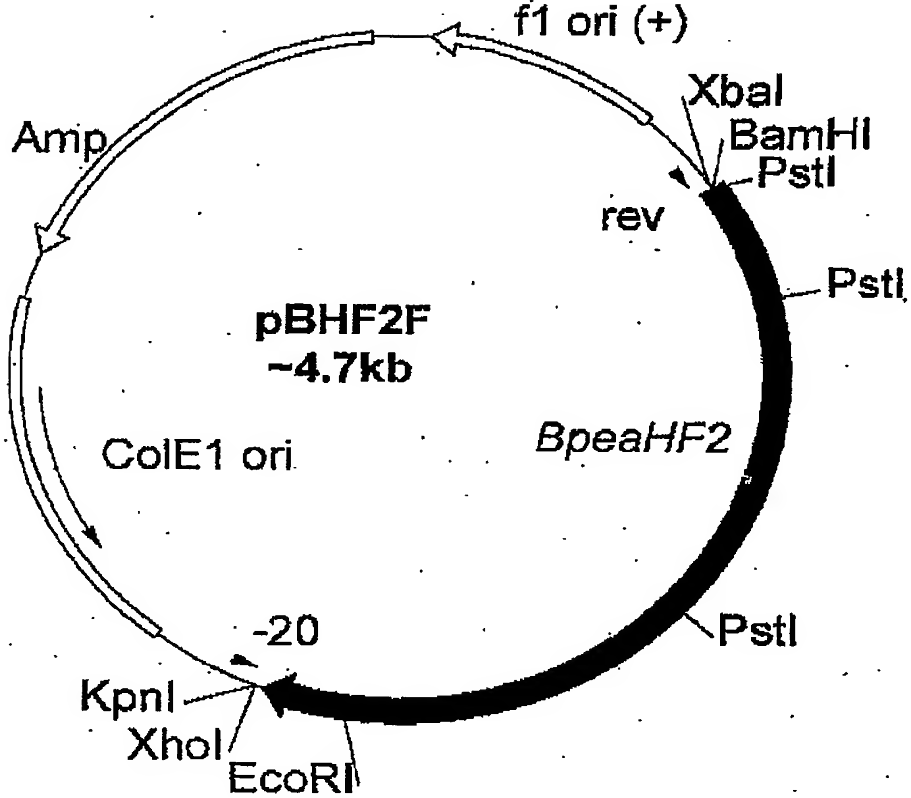
Figure 41



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb (XhoI/NotI) blunted fragment containing *CaMV 35S: Kenn#31: ocs 3'* gene from pCGP2236

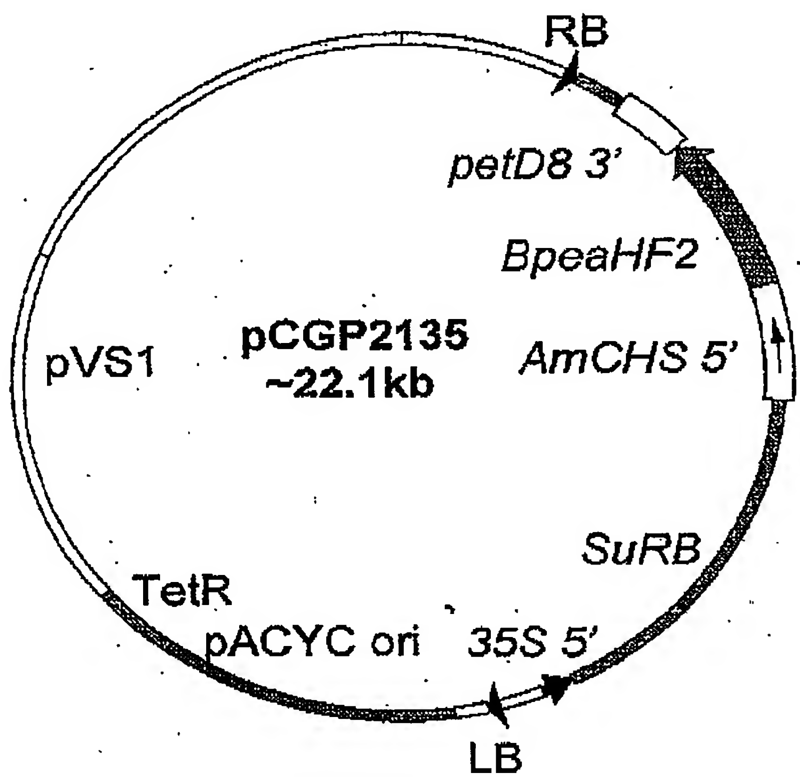
Figure 42



Replicon: pBHF2 BamHI/PstI 4.5kb vector + partial *BpeaHF2* insert (backbone = pBluescript SK II (+) vector)

Insert: ~200bp BamHI/PstI fragment from PCR using pBHF2 as template (5' fragment of butterfly pea *F3'5'H* cDNA (*BpeaHF2*) *from Clitoria ternatea* including putative initiating codon (ATG))

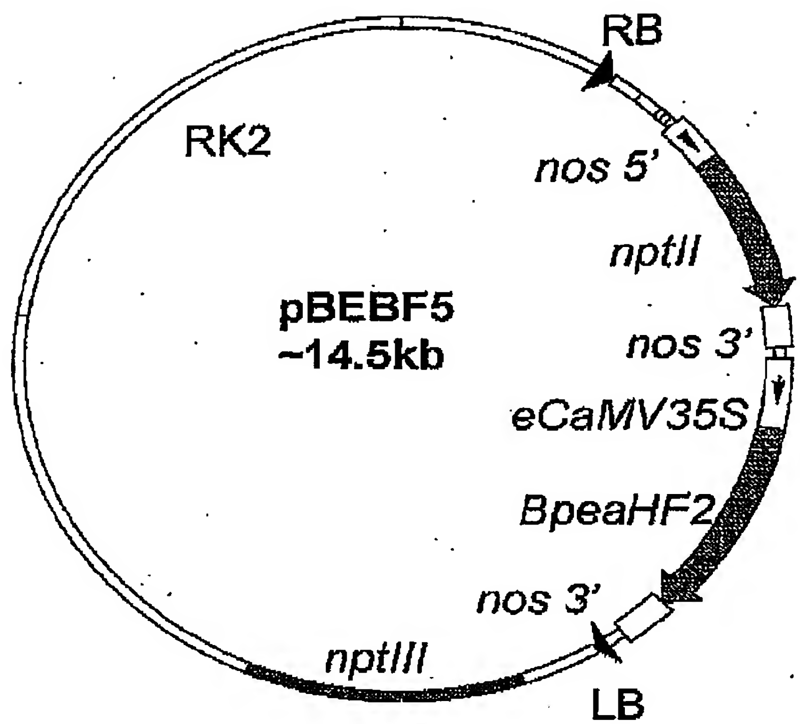
Figure 43



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': BpeaHF2: petD8 3'* gene from pCGP2133

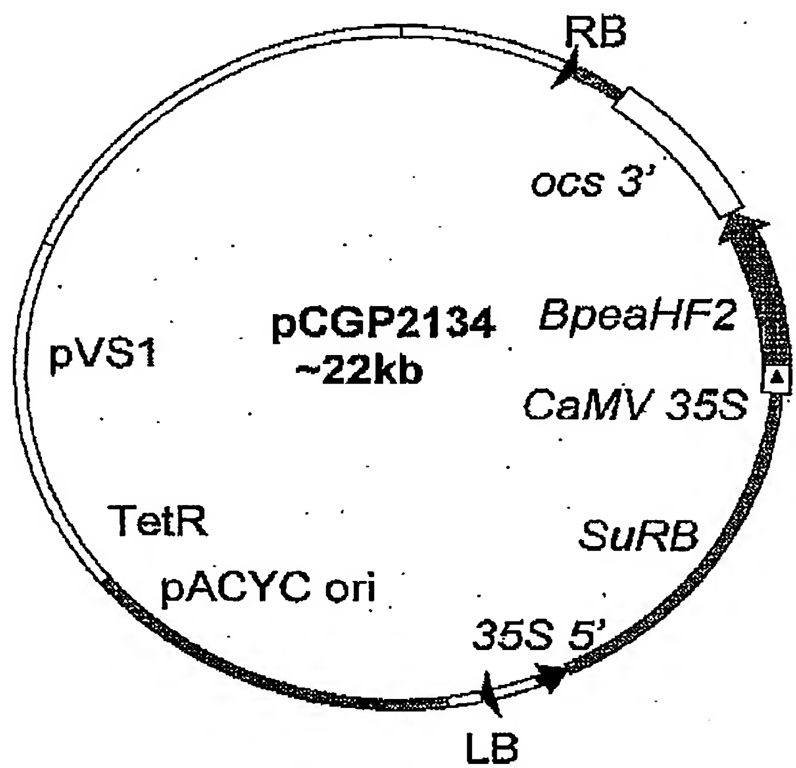
Figure 44



Replicon: ~12.8kb pBE2113-GUSs BamHI/SalI (pBI121 backbone)

Insert: ~1.7kb BamHI/XhoI fragment containing *Clitoria F3'5'H BpeaHF2* cDNA clone from pBHF2F

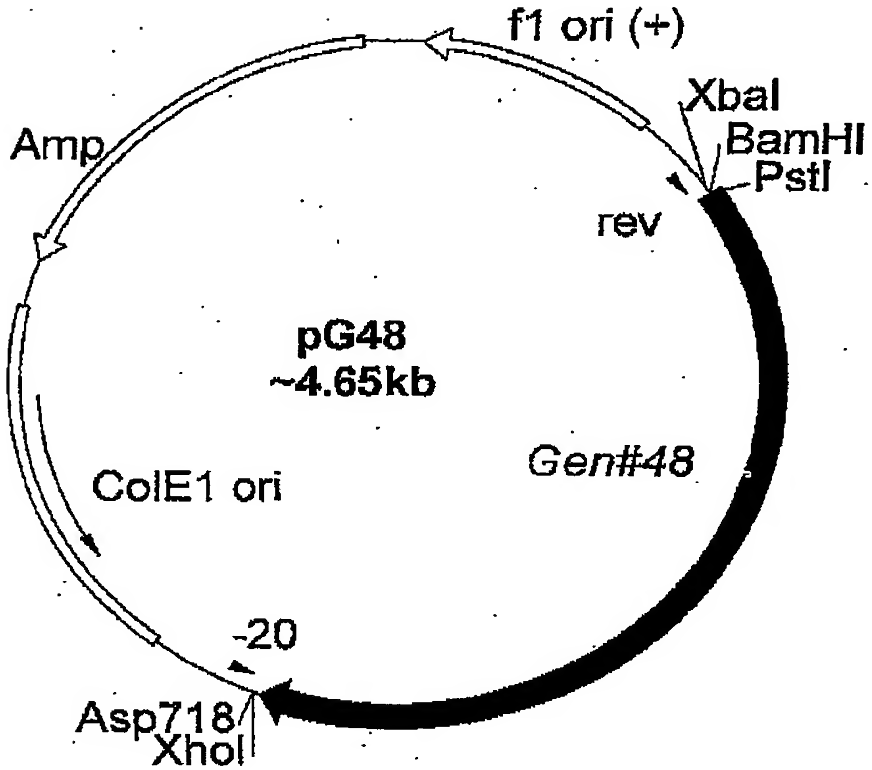
Figure 45



Replicon: ~18.4kb Asp718 (blunted) pCGP1988 vector

Insert: ~3.6kb (XhoI/XbaI) blunted fragment containing *CaMV 35S: BpeaHF2: ocs 3'* gene from pCGP2132

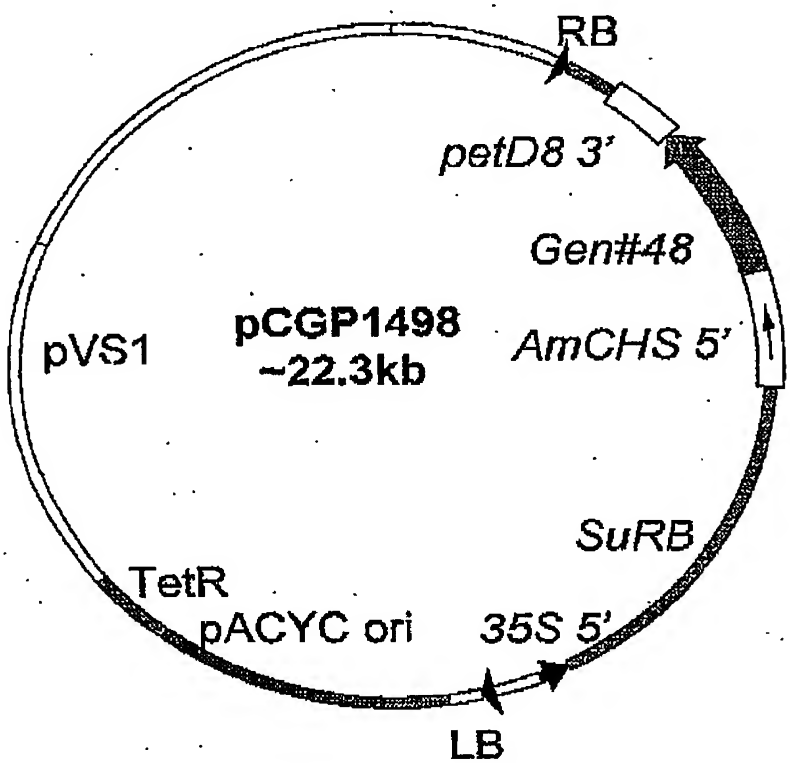
Figure 46



Replicon: pBluescript SK II (+) vector 2.95kb

Insert: ~1.7kb *F3'5'H Gen#48* cDNA from *Gentiana triflora*

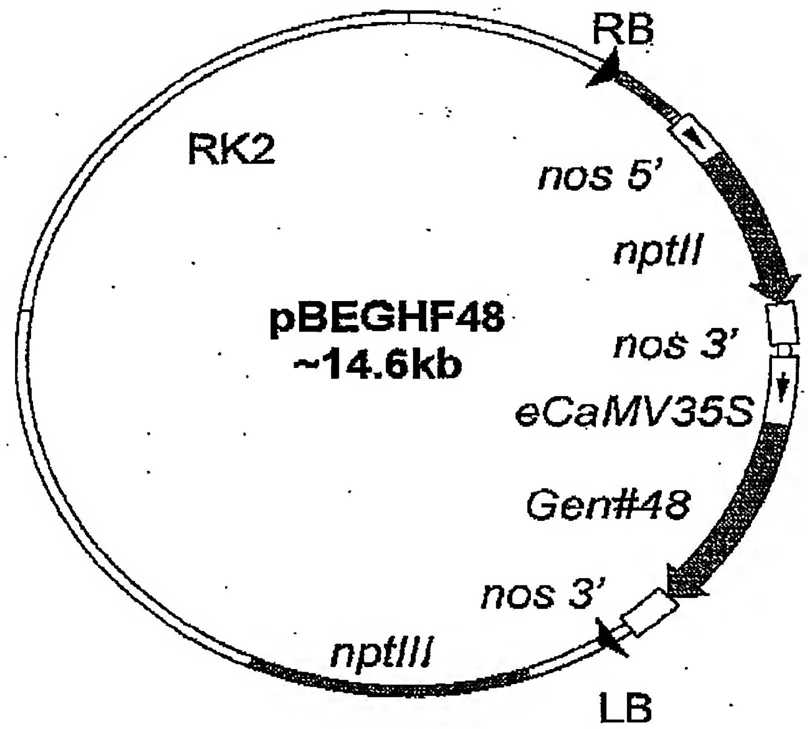
Figure 47



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.6kb NotI (blunted)/ EcoRV fragment containing *AmCHS 5': Gen#48: petD8 3'* gene from pCGP1496

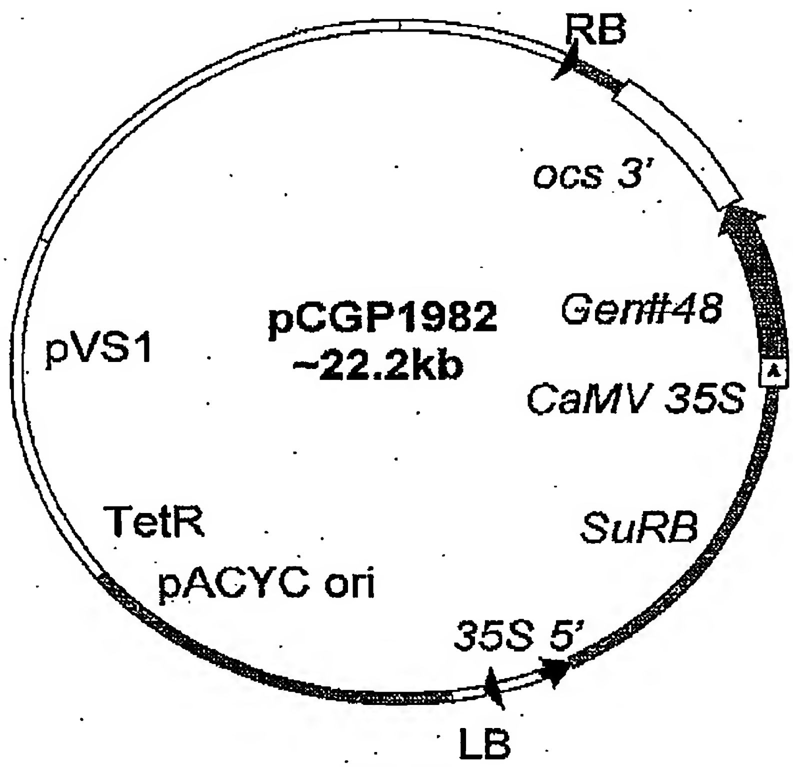
Figure 48



Replicon: ~12.8kb pBE2113-GUSs BamHI/SalI (pBI121 backbone)

Insert: ~1.8kb BamHI/XhoI fragment containing *gentian F3'5'H* (*Genn#48*) cDNA clone from pG48

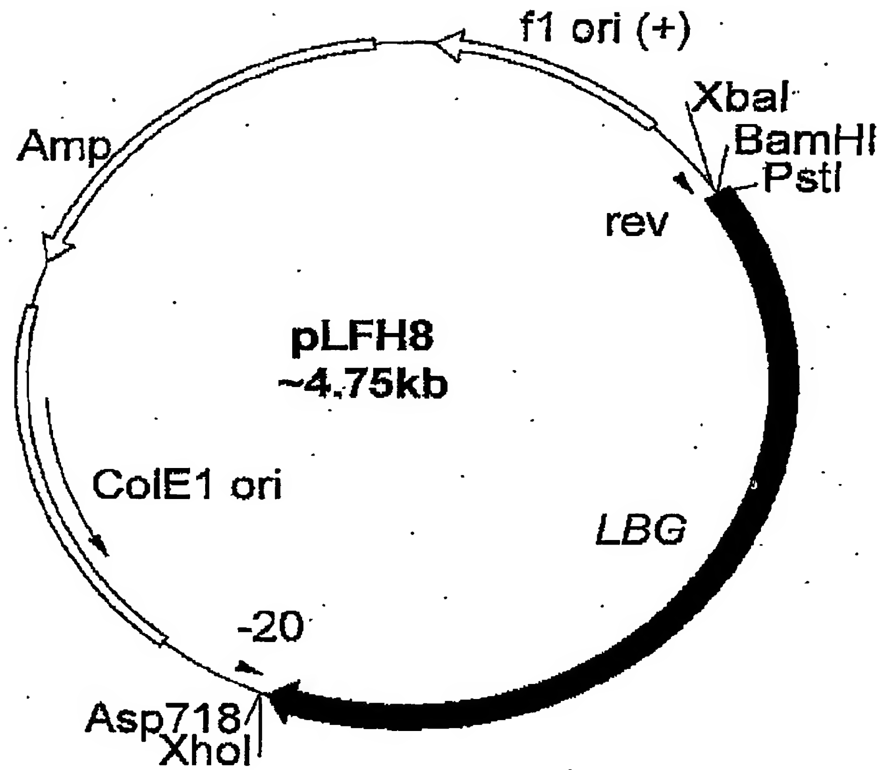
Figure 49



Replicon: ~18.7kb Asp718 (blunted) pWTT2132 vector

Insert: ~3.6kb (XhoI/XbaI) blunted fragment containing *CaMV 35S: Gen#48: ocs 3'* gene from pCGP1981

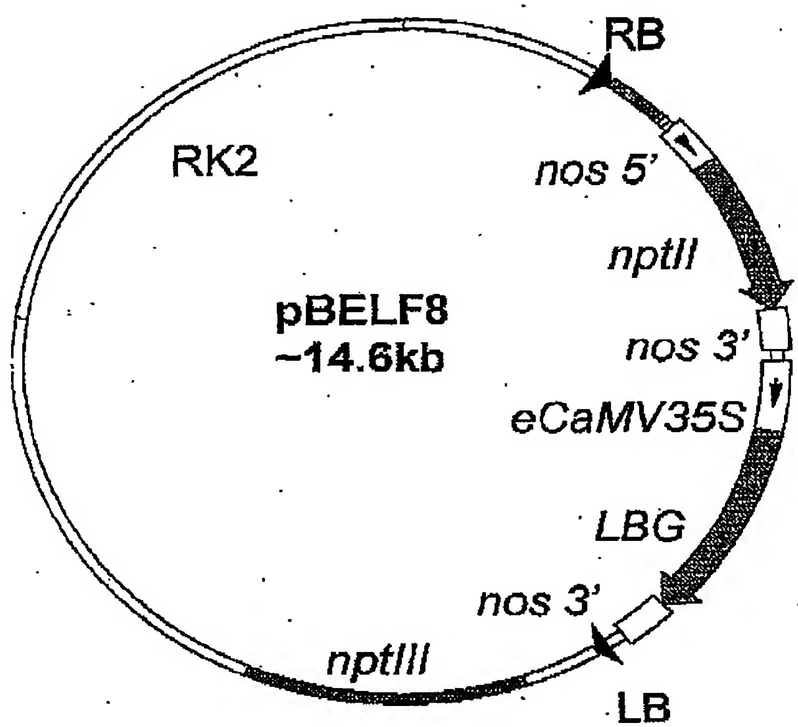
Figure 50



Replicon: pBluescript SK II (+) vector 2.95kb

Insert: ~1.8kb *lavender F3'5'H LBG* cDNA from *Lavendula nil*

Figure 51



Replicon: ~12.8kb pBE2113-GUSs BamHI/SalI (pBI121 backbone)

Insert: ~1.8kb BamHI/XhoI fragment containing *lavender F3'5'H* (*LBG*) cDNA clone from pLHF8

Figure 52

SEQUENCE LISTING

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       Brugliera, Filippa (US only)
       Tanaka, Yoshikazu (US only)
       Mason, John (US only)

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    50                  55                  60

Gly Ala Ile Met Tyr Leu Lys Val Gly Thr Cys Gly Met Ala Val Ala
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Ser Thr Pro Asp Ala Ala Lys Ala Phe Leu Lys Thr Leu Asp Ile Asn
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Gly Ala Ile Met Tyr Leu Lys Val Gly Thr Cys Gly Met Val Val Ala
65                  70                  75                  80

Ser Thr Pro Asp Ala Ala Lys Ala Phe Leu Lys Thr Leu Asp Leu Asn
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Phe Ser Asn Arg Pro Pro Asn Ala Gly Ala Thr His Leu Ala Tyr Gly
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Arg Ala Ile Cys Lys Glu Thr Phe Arg Lys His Pro Ser Thr Pro Leu
        355                 360                 365

Asn Leu Pro Arg Ile Ser Asn Glu Pro Cys Ile Val Asp Gly Tyr Tyr
    370                 375                 380

Ile Pro Lys Asn Thr Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg
385                 390                 395                 400

Asp Pro Glu Val Trp Glu Asn Pro Leu Glu Phe Tyr Pro Glu Arg Phe
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Gly Ile Val Met Val Glu Tyr Ile Leu Gly Thr Leu Val His Ser Phe
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Asp Trp Lys Leu Pro Ser Glu Val Ile Glu Leu Asn Met Glu Glu Ala
465                 470                 475                 480

Phe Gly Leu Ala Leu Gln Lys Ala Val Pro Leu Glu Ala Met Val Thr
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Pro Arg Leu Pro Ile Asp Val Tyr Ala Pro Leu Ala
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&lt;400&gt;  10

Met Ala Ile Pro Val Thr Asp Leu Ala Val Ala Val Ile Leu Phe Leu
1               5                   10                  15

Ile Thr Arg Phe Leu Val Arg Ser Leu Phe Lys Lys Pro Thr Gly Pro
            20                  25                  30

Leu Pro Pro Gly Pro Ser Gly Trp Pro Leu Val Gly Ala Leu Pro Leu
        35                  40                  45

Leu Gly Ala Met Pro His Val Thr Leu Ala Asn Leu Ala Lys Lys Tyr
    50                  55                  60

Gly Pro Ile Met Tyr Leu Lys Met Gly Thr Cys Asp Met Val Val Ala
65                  70                  75                  80

Ser Thr Pro Asp Ser Ala Arg Ala Phe Leu Lys Thr Leu Asp Leu Asn
                85                  90                  95

Phe Ser Asp Arg Pro Pro Asn Ala Gly Ala Thr His Leu Ala Tyr Gly
            100                 105                 110

Ala Gln Asp Leu Val Phe Ala Lys Tyr Gly Pro Arg Trp Lys Thr Leu
        115                 120                 125

Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly Lys Ala Leu Asp Asp
    130                 135                 140

Trp Ala His Val Arg Ala Asn Glu Leu Gly His Met Leu Asn Ala Met
145                 150                 155                 160
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Cys Glu Ala Ser Arg Cys Gly Glu Pro Val Val Leu Ala Glu Met Leu
165 170 175

Thr Tyr Ala Met Ala Asn Met Ile Gly Gln Val Ile Leu Ser Arg Arg
180 185 190

Val Phe Val Thr Lys Gly Thr Glu Ser Asn Glu Phe Lys Asp Met Val
195 200 205

Val Glu Leu Met Thr Ser Ala Gly Tyr Phe Asn Ile Gly Asp Phe Ile
210 215 220

Pro Ser Ile Ala Trp Met Asp Leu Gln Gly Ile Glu Arg Gly Met Lys
225 230 235 240

Lys Leu His Thr Lys Phe Asp Val Leu Leu Thr Lys Met Met Lys Glu
245 250 255

His Arg Ala Thr Ser His Glu Arg Glu Gly Lys Ser Asp Phe Leu Asp
260 265 270

Val Leu Leu Glu Glu Cys Glu Asn Thr Asn Gly Glu Lys Leu Asn Val
275 280 285

Thr Asn Val Lys Ala Val Leu Leu Asn Leu Phe Thr Ala Gly Thr Asp
290 295 300

Thr Ser Ser Ser Ile Ile Glu Trp Ala Leu Thr Glu Met Met Lys Asn
305 310 315 320

Pro Thr Ile Leu Lys Lys Thr Gln Glu Glu Met Asp Arg Val Ile Gly
325 330 335

Arg Asp Arg Arg Leu Leu Glu Ser Asp Val Ser Lys Leu Pro Tyr Leu
340 345 350

Gln Ala Ile Ala Lys Glu Thr Tyr Arg Lys His Pro Ser Thr Pro Leu
355 360 365

Asn Leu Pro Arg Ile Ala Ile Gln Ala Cys Glu Val Asp Gly Tyr Tyr
370 375 380

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Ile Pro Lys Asp Thr Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg
385                 390                 395                 400

Asp Pro Ser Val Trp Glu Asn Pro Ser Glu Phe Ser Pro Glu Arg Phe
                405                 410                 415

Leu Ser Glu Glu Asn Gly Lys Ile Ser Pro Gly Gly Asn Asp Phe Glu
            420                 425                 430

Leu Ile Pro Phe Gly Ala Gly Arg Arg Ile Cys Ala Gly Thr Arg Met
        435                 440                 445

Gly Met Val Leu Val Ser Tyr Ile Leu Gly Thr Leu Val His Ser Phe
    450                 455                 460

Asp Trp Lys Leu Pro Asn Gly Val Ser Glu Ile Asn Met Asp Glu Ser
465                 470                 475                 480

Phe Gly Leu Ala Leu Gln Lys Ala Val Pro Leu Ser Ala Thr Val Ser
                485                 490                 495

Pro Arg Leu Ala Pro Ser Ala Tyr Val Ile
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cggttggcoc ttggtgggcg ccctccctct cctaggcgcc atgcctcacg tcgcactagc     180

caaactcgct aagaagtatg gtccgatcat gcacctaaaa atgggcacgt gcgacatggt     240

ggtcgcgtcc accccogagt cggctcgagc cttcctcaaa acgctagacc tcaacttctc     300

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cgccaagtac ggtccgaggt ggaagacttt aagaaaattg agcaacctcc acatgctagg     420
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<400> 12

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Leu Pro Pro Gly Pro Leu Gly Trp Pro Leu Val Gly Ala Leu Pro Leu
        35                  40                  45

Leu Gly Ala Met Pro His Val Ala Leu Ala Lys Leu Ala Lys Lys Tyr
    50                  55                  60

Gly Pro Ile Met His Leu Lys Met Gly Thr Cys Asp Met Val Val Ala
65                  70                  75                  80

Ser Thr Pro Glu Ser Ala Arg Ala Phe Leu Lys Thr Leu Asp Leu Asn
                85                  90                  95

Phe Ser Asn Arg Pro Pro Asn Ala Gly Ala Ser His Leu Ala Tyr Gly
            100                 105                 110

Ala Gln Asp Leu Val Phe Ala Lys Tyr Gly Pro Arg Trp Lys Thr Leu
        115                 120                 125

Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly Lys Ala Leu Asp Asp
    130                 135                 140

Trp Ala Asn Val Arg Val Thr Glu Leu Gly His Met Leu Lys Ala Met
145                 150                 155                 160

Cys Glu Ala Ser Arg Cys Gly Glu Pro Val Val Leu Ala Glu Met Leu
                165                 170                 175

Thr Tyr Ala Met Ala Asn Met Ile Gly Gln Val Ile Leu Ser Arg Arg
            180                 185                 190

Val Phe Val Thr Lys Gly Thr Glu Ser Asn Glu Phe Lys Asp Met Val
        195                 200                 205

Val Glu Leu Met Thr Ser Ala Gly Tyr Phe Asn Ile Gly Asp Phe Ile
    210                 215                 220

Pro Ser Ile Ala Trp Met Asp Leu Gln Gly Ile Glu Arg Gly Met Lys
225                 230                 235                 240
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Lys Leu His Thr Lys Phe Asp Val Leu Leu Thr Lys Met Val Lys Glu
                245                 250                 255

His Arg Ala Thr Ser His Glu Arg Lys Gly Lys Ala Asp Phe Leu Asp
            260                 265                 270

Val Leu Leu Glu Glu Cys Asp Asn Thr Asn Gly Glu Lys Leu Ser Ile
        275                 280                 285

Thr Asn Ile Lys Ala Val Leu Leu Asn Leu Phe Thr Ala Gly Thr Asp
    290                 295                 300

Thr Ser Ser Ser Ile Ile Glu Trp Ala Leu Thr Glu Met Ile Lys Asn
305                 310                 315                 320

Pro Thr Ile Leu Lys Lys Ala Gln Glu Glu Met Asp Arg Val Ile Gly
                325                 330                 335

Arg Asp Arg Arg Leu Leu Glu Ser Asp Ile Ser Ser Leu Pro Tyr Leu
            340                 345                 350

Gln Ala Ile Ala Lys Glu Thr Tyr Arg Lys His Pro Ser Thr Pro Leu
        355                 360                 365

Asn Leu Pro Arg Ile Ala Ile Gln Ala Cys Glu Val Asp Gly Tyr Tyr
    370                 375                 380

Ile Pro Lys Asp Ala Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg
385                 390                 395                 400

Asp Pro Asn Val Trp Glu Asn Pro Leu Glu Phe Leu Pro Glu Arg Phe
                405                 410                 415

Leu Ser Glu Glu Asn Gly Lys Ile Asn Pro Gly Gly Asn Asp Phe Lys
            420                 425                 430

Leu Ile Pro Phe Gly Ala Gly Arg Arg Ile Cys Ala Gly Thr Arg Met
        435                 440                 445

Gly Met Val Leu Val Ser Tyr Ile Leu Gly Thr Leu Val His Ser Phe
    450                 455                 460
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Asp Trp Lys Leu Pro Asn Gly Val Ala Glu Leu Asn Met Asp Glu Ser
465                 470                 475                 480

Phe Gly Leu Ala Leu Gln Lys Ala Val Pro Leu Ser Ala Leu Val Ser
                485                 490                 495

Pro Arg Leu Ala Ser Asn Pro Tyr Ala Thr
            500                 505


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cgccctagca aaaatggcca aaacttacgg cccgatcatg tacttgaaaa tgggcacagt     240

cggcatggtc gtggcgtcca cgccagacgc ggcgcgggcg ttcctaaaaa cccacgacgc     300

taatttctcg aaccggccgg tcaacgcggg tgccaccatc ctggcatacа atgcccagga     360

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<213> salvia

<400> 14

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Leu Val Val Ala Ser Leu Leu Tyr Ile Leu Ile Arg Met Phe Ile Ser
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Lys Leu Ser Thr Thr Gly His Pro Leu Pro Pro Gly Pro Arg Gly Phe
35 40 45

Leu Val Val Gly Ser Leu Pro Leu Leu Gly Asp Met Pro His Val Ala
50 55 60

Leu Ala Lys Met Ala Lys Thr Tyr Gly Pro Ile Met Tyr Leu Lys Met
65 70 75 80

Gly Thr Val Gly Met Val Val Ala Ser Thr Pro Asp Ala Ala Arg Ala
85 90 95

Phe Leu Lys Thr His Asp Ala Asn Phe Ser Asn Arg Pro Val Asn Ala
100 105 110

Gly Ala Thr Ile Leu Ala Tyr Asn Ala Gln Asp Met Val Phe Ala Pro
115 120 125

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Tyr Gly Pro Lys Trp Arg Leu Leu Arg Lys Leu Ser Ser Leu His Met
    130                 135                 140

Leu Gly Ser Lys Ala Leu Glu Glu Trp Ala Asp Val Arg Thr Ser Glu
145                 150                 155                 160

Val Gly His Met Leu Ala Ala Met His Glu Ala Ser Arg Leu Gly Glu
                165                 170                 175

Ala Val Gly Leu Pro Glu Met Leu Val Tyr Ala Thr Ala Asn Met Ile
            180                 185                 190

Gly Gln Val Ile Leu Ser Arg Arg Val Phe Val Thr Lys Gly Lys Glu
        195                 200                 205

Met Asn Glu Phe Lys Glu Met Val Val Glu Leu Met Thr Thr Ala Gly
    210                 215                 220

Tyr Phe Asn Ile Gly Asp Phe Ile Pro Trp Leu Ala Trp Met Asp Leu
225                 230                 235                 240

Gln Gly Ile Glu Arg Gly Met Lys Lys Leu His Lys Lys Trp Asp Arg
                245                 250                 255

Leu Ile Gly Lys Met Leu Asp Asp Arg Leu Lys Ser Thr Tyr Lys Arg
            260                 265                 270

Asn Asp Lys Pro Asp Leu Leu Asp Ser Leu Leu Ala Asn His Asp Asp
        275                 280                 285

Glu Ser Lys Asp Asp Asp Glu Asp Cys Lys Leu Thr Thr Thr Asn Ile
    290                 295                 300

Lys Ala Leu Leu Leu Asn Leu Phe Thr Ala Gly Thr Asp Thr Ser Ser
305                 310                 315                 320

Ser Ile Ile Glu Trp Ala Leu Ala Glu Met Ile Lys Asn Pro Ser Ile
                325                 330                 335

Gln Lys Arg Ala His Gln Glu Met Asp Arg Val Ile Gly Arg Glu Arg
            340                 345                 350
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Arg Leu Leu Glu Ser Asp Ile Pro Asn Leu Pro Tyr Leu Lys Ala Ile
355 360 365

Cys Lys Glu Ala Tyr Arg Lys His Pro Ser Thr Pro Leu Asn Leu Pro
370 375 380

Arg Ile Ser Thr Asp Ala Cys Val Val Asp Gly Tyr His Ile Pro Lys
385 390 395 400

Asn Thr Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg Asp Pro Asp
405 410 415

Val Trp Glu Asn Pro Leu Asp Phe Asn Pro Asp Arg Phe Met Ser Gly
420 425 430

Leu Gln Gly Ile Glu Pro Gly Gly Asn His Phe Glu Leu Ile Pro Phe
435 440 445

Gly Ala Gly Arg Arg Ile Cys Ala Gly Ser Arg Met Gly Ile Val Ile
450 455 460

Val Glu Tyr Leu Leu Ala Thr Leu Val His Ser Phe Glu Trp Asp Leu
465 470 475 480

Pro Ala Gly Ser Ala Glu Met Asp Met Glu Glu Val Phe Gly Leu Ala
485 490 495

Leu Gln Lys Ala Val Pro Leu Ala Ala Arg Leu Thr Pro Arg Leu Pro
500 505 510

Ser His Cys Tyr Ala Pro Pro Ser
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<212> DNA
<213> salvia

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ctctgccccc ggggccgagg ggctttccag tggtgggctc ccttcccttg ctgggcgaca 180

tgccacatgt tgccctagca aaaatggcca aaacttatgg cccgatcatg tacttgaaaa 240

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atgcccagga catggtgttt gccccgtacg gccccaagtg gagattgctg aggaagctga     420
gcagtctcca catgctgggg agcaaggccc tggaggagtg ggccgacgtc cggacctcgg     480
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tgccggagat gctggtgtac gcgacggcga acatgatcgg gaaggtgata ttgagccgga     600
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ctgtaccact tgctgctagg ctcactccta ggttgccttc acattgctat gcacctcctt    1560
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<210> 16
<211> 518
<212> PRT
<213> salvia

<400> 16

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Ala Ser Leu Leu Tyr Ile Leu Ile Arg Met Phe Ile Ser Lys Leu Ser
            20                  25                  30

Thr Pro Gly His Pro Leu Pro Pro Gly Pro Arg Gly Phe Pro Val Val
        35                  40                  45

Gly Ser Leu Pro Leu Leu Gly Asp Met Pro His Val Ala Leu Ala Lys
    50                  55                  60

Met Ala Lys Thr Tyr Gly Pro Ile Met Tyr Leu Lys Met Gly Thr Val
65                  70                  75                  80

Gly Met Val Val Ala Ser Thr Pro Asp Ala Ala Arg Ala Phe Leu Lys
                85                  90                  95

Thr Gln Asp Ala Asn Phe Ser Asn Arg Pro Val Asn Ala Gly Ala Thr
            100                 105                 110

Ile Leu Ala Tyr Asn Ala Gln Asp Met Val Phe Ala Pro Tyr Gly Pro
        115                 120                 125

Lys Trp Arg Leu Leu Arg Lys Leu Ser Ser Leu His Met Leu Gly Ser
    130                 135                 140

Lys Ala Leu Glu Glu Trp Ala Asp Val Arg Thr Ser Glu Val Gly His
145                 150                 155                 160

Met Leu Ala Ala Met His Glu Ala Ser Arg Leu Asp Glu Ala Val Gly
                165                 170                 175

Leu Pro Glu Met Leu Val Tyr Ala Thr Ala Asn Met Ile Gly Lys Val
            180                 185                 190

Ile Leu Ser Arg Arg Val Phe Val Thr Lys Gly Lys Glu Met Asn Glu
        195                 200                 205

Phe Lys Glu Met Val Val Glu Leu Met Thr Thr Ala Gly Tyr Phe Asn
    210                 215                 220

Ile Gly Asp Phe Ile Pro Trp Leu Ala Trp Met Asp Leu Gln Gly Ile
225                 230                 235                 240
```

```
Glu Arg Gly Met Lys Lys Leu His Lys Lys Trp Asp Arg Leu Ile Gly
                245                 250                 255

Lys Met Leu Asp Asp Arg Leu Lys Ser Thr Tyr Lys Arg Asn Asp Lys
            260                 265                 270

Pro Asp Leu Leu Asp Ser Leu Leu Ala Asn His Asp Asp Glu Ser Lys
        275                 280                 285

Asp Asp Asp Glu Asp Cys Lys Leu Thr Thr Thr Asn Ile Lys Ala Leu
    290                 295                 300

Leu Leu Asn Leu Phe Thr Ala Gly Thr Asp Thr Ser Ser Ser Ile Ile
305                 310                 315                 320

Glu Trp Ala Leu Ala Glu Met Ile Lys Asn Pro Ser Ile Gln Lys Arg
                325                 330                 335

Ala His Gln Glu Met Asp Arg Val Ile Gly Arg Glu Arg Arg Leu Leu
            340                 345                 350

Glu Ser Asp Ile Pro Asn Leu Pro Tyr Leu Lys Ala Ile Cys Lys Glu
        355                 360                 365

Ala Tyr Arg Lys His Pro Ser Thr Pro Leu Asn Leu Pro Arg Ile Ser
    370                 375                 380

Thr Asp Ala Cys Val Val Asp Gly Tyr His Ile Pro Lys Asn Thr Arg
385                 390                 395                 400

Leu Ser Val Asn Ile Trp Ala Ile Gly Arg Asp Pro Asp Val Trp Glu
                405                 410                 415

Asn Pro Leu Asp Phe Asn Pro Asp Arg Phe Met Ser Gly Leu Gln Gly
            420                 425                 430

Ile Glu Pro Gly Gly Asn His Phe Glu Leu Ile Pro Phe Gly Ala Gly
        435                 440                 445

Arg Arg Ile Cys Ala Gly Ser Arg Met Gly Ile Val Ile Val Glu Tyr
    450                 455                 460
```

```
Leu Leu Ala Thr Leu Val His Ser Phe Glu Trp Asp Leu Pro Ala Gly
465                 470                 475                 480

Ser Ala Glu Met Asp Met Glu Glu Val Phe Gly Leu Ala Leu Gln Lys
                485                 490                 495

Ala Val Pro Leu Ala Ala Arg Leu Thr Pro Arg Leu Pro Ser His Cys
            500                 505                 510

Tyr Ala Pro Pro Ser Ile
        515


&lt;210&gt;  17
&lt;211&gt;  1730
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&lt;213&gt;  sollya

&lt;220&gt;
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aagaccatgg cctattgtag gaaacctccc acaccttggc accaagccac accactccat     180

agctgccatg gctcggaaat acggtcccct cctgcacctc cgcatgggca tcgtgcacgt     240

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tgcaccctac ggtccaaggt ggcgcatgtt gaggaaaatt tgtgcccttc atatattctc     420

cgctaaggct ctcgatgatt ttcatcgcgt gcgtgaggag gaggttgcca tactcgcgag     480

gaccctagca cacgcaggcc aaaagccggt gaatttgggg cagttgttct ctacgtgtaa     540

tgctaatgcg ctatcagtgc tgatgctagg caggaggttg ttcagcacag aagttgattc     600

aaaagcatat gatttcaaac aaatggtggt ggagctgatg actctagccg gtgagtttaa     660

cgtcagtgat ttcatcccac ccctcgagtg gctagacttg caaggcgtgg cagcgaaaat     720

gaagaacgtg cacaatcgat tcgatgcgtt tctgaatgta attttggagg agcataagct     780

gaaacttaat aatagtggac atggggaaca aaaacatatg gacttgttga gtacgttgat     840

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aaactgagta cattcatgaa ctgacccaga agctgtcaga tgtcgtctta tattgcctta   1620
tgtagtgcga cocttgtgtg tttttatgt attgttttgt acaaggttga agcccgtgcg   1680
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<210>  18
<211>  521
<212>  PRT
<213>  sollya

<400>  18

Met Ala Thr Thr Leu Glu Phe Ile Leu Cys Phe Thr Ile Thr Ala Leu
1               5                   10                  15

Pro Phe Leu Tyr Cys Ile Leu Asn Met Arg Ile Leu Leu Asn Arg His
            20                  25                  30

Pro Arg Ser Leu Pro Pro Gly Pro Arg Pro Trp Pro Ile Val Gly Asn
        35                  40                  45

Leu Pro His Leu Gly Thr Lys Pro His His Ser Ile Ala Ala Met Ala
    50                  55                  60

Arg Lys Tyr Gly Pro Leu Leu His Leu Arg Met Gly Ile Val His Val
65                  70                  75                  80
```

Val Val Ala Ala Ser Ala Asp Val Ala Ala Gln Phe Leu Lys Asn Asp
85 90 95

Ala Asn Phe Ser Ser Arg Pro Pro Asn Ser Gly Ala Lys His Met Ala
100 105 110

Tyr Asn Tyr His Asp Met Val Phe Ala Pro Tyr Gly Pro Arg Trp Arg
115 120 125

Met Leu Arg Lys Ile Cys Ala Leu His Ile Phe Ser Ala Lys Ala Leu
130 135 140

Asp Asp Phe His Arg Val Arg Glu Glu Glu Val Ala Ile Leu Ala Arg
145 150 155 160

Thr Leu Ala His Ala Gly Gln Lys Pro Val Asn Leu Gly Gln Leu Phe
165 170 175

Ser Thr Cys Asn Ala Asn Ala Leu Ser Val Leu Met Leu Gly Arg Arg
180 185 190

Leu Phe Ser Thr Glu Val Asp Ser Lys Ala Tyr Asp Phe Lys Gln Met
195 200 205

Val Val Glu Leu Met Thr Leu Ala Gly Glu Phe Asn Val Ser Asp Phe
210 215 220

Ile Pro Pro Leu Glu Trp Leu Asp Leu Gln Gly Val Ala Ala Lys Met
225 230 235 240

Lys Asn Val His Asn Arg Phe Asp Ala Phe Leu Asn Val Ile Leu Glu
245 250 255

Glu His Lys Leu Lys Leu Asn Asn Ser Gly His Gly Glu Gln Lys His
260 265 270

Met Asp Leu Leu Ser Thr Leu Ile Leu Leu Lys Asp Asp Ala Asp Ser
275 280 285

Glu Gly Gly Lys Leu Thr Asp Thr Glu Ile Lys Ala Leu Leu Leu Asn
290 295 300

```
Leu Phe Ser Ala Gly Thr Asp Thr Ser Ser Ser Thr Ile Glu Trp Val
305                 310                 315                 320

Ile Ala Glu Leu Ile Arg Asn Pro Lys Ile Leu Ala Gln Ala Gln Arg
                325                 330                 335

Glu Leu Asp Leu Val Val Gly Pro Asn Arg Leu Val Thr Asp Leu Asp
            340                 345                 350

Leu Lys Gln Leu Thr Tyr Leu Gln Ala Ile Val Lys Glu Thr Phe Arg
        355                 360                 365

Leu His Pro Ala Thr Pro Leu Ser Leu Pro Arg Ile Ala Thr Glu Ser
    370                 375                 380

Cys Glu Ile Asn Gly Phe Tyr Ile Pro Lys Gly Ser Thr Leu Leu Val
385                 390                 395                 400

Asn Ile Trp Ala Ile Gly Arg Asp Pro Asn Thr Trp Ala Glu Pro Leu
                405                 410                 415

Val Phe Arg Pro Glu Arg Phe Leu Ser Asp Gly Glu Ser Pro Asn Val
            420                 425                 430

Asp Val Lys Gly Arg Asn Phe Glu Leu Ile Pro Phe Gly Ala Gly Arg
        435                 440                 445

Arg Ile Cys Ala Gly Met Asn Phe Gly Leu Arg Met Val Gln Leu Val
    450                 455                 460

Thr Ala Thr Leu Ile His Ala Phe Asn Trp Glu Leu Pro Glu Gly Glu
465                 470                 475                 480

Leu Pro Glu Asn Met Asn Met Glu Glu Asp Tyr Gly Ile Ser Leu Gln
                485                 490                 495

Arg Thr Val Pro Leu Val Val His Pro Lys Pro Arg Leu Asp His Glu
            500                 505                 510

Val Tyr Gln Ser His Gly Val Val Asn
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<213> petunia

<400> 19
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<210> 20
<211> 1748
<212> DNA
<213> clitoria

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aaaaaaaa                                                            1748

<210>  21
<211>  525
<212>  PRT
<213>  clitoria

<400>  21

Met Phe Leu Leu Arg Glu Ile Gly Val Ser Ile Leu Ile Phe Met Ile
1               5                   10                  15

Thr His Leu Val Ile Arg Leu Val Leu Lys Glu Lys Glu Gln Arg Lys
            20                  25                  30

Leu Pro Pro Gly Pro Lys Gly Trp Pro Ile Val Gly Ala Leu Pro Leu
        35                  40                  45

Met Gly Ser Met Pro His Val Thr Leu Ser Glu Met Ala Lys Lys Tyr
    50                  55                  60

Gly Pro Val Met Tyr Leu Lys Met Gly Thr Asn Asn Met Ala Val Ala
65                  70                  75                  80

Ser Thr Pro Ser Ala Ala Arg Ala Phe Leu Lys Thr Leu Asp Leu Asn
                85                  90                  95

Phe Ser Asn Arg Pro Pro Asn Ala Gly Ala Thr His Leu Ala Tyr Asp
            100                 105                 110

Ala Gln Asp Met Val Phe Ala Asp Tyr Gly Ser Arg Trp Lys Leu Leu
        115                 120                 125
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Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly Lys Ala Leu Glu Glu
130 135 140

Trp Ser Gln Val Arg Glu Ile Glu Met Gly His Met Leu Arg Ala Met
145 150 155 160

Tyr Asp Cys Ser Gly Gly Gly Asp Gly Asn Asn Asp Asn Asp Gly Asn
165 170 175

Lys Lys Lys Gly Thr Arg His Glu Pro Ile Val Val Ala Glu Met Leu
180 185 190

Thr Tyr Ala Met Ala Asn Met Ile Gly Pro Ser Asp Leu Glu Pro Ser
195 200 205

Cys Ile Pro Arg Gln Arg Val Arg Asn Arg Thr Ser Leu Arg Thr Trp
210 215 220

Trp Phe Lys Leu Met Thr Val Ala Gly Tyr Phe Asn Ile Gly Asp Phe
225 230 235 240

Phe Pro Phe Leu Ala Arg Arg Arg Arg Gln Gly Ile Glu Arg Gly Met
245 250 255

Lys Thr Leu His Asn Lys Lys Asp Asp Leu Leu Thr Thr Met Ile His
260 265 270

Glu His Val Ala Ser Ala His Lys Arg Lys Gly Lys Pro Pro Phe Leu
275 280 285

Asp Val Leu Met Ala His His Thr Asn Glu Ser His Glu Leu Ser Leu
290 295 300

Thr Asn Ile Lys Ala Leu Leu Leu Asn Leu Phe Thr Ala Gly Thr Asp
305 310 315 320

Thr Ser Ser Ser Ile Ile Glu Trp Ala Leu Ala Glu Met Leu Ile Asn
325 330 335

Pro Lys Ile Met Lys Lys Val His Glu Glu Met Asp Lys Val Ile Gly
340 345 350

```
Lys Asp Arg Arg Leu Lys Glu Ser Asp Ile Glu Asn Leu Pro Tyr Leu
        355                 360                 365

Gln Ala Ile Cys Lys Glu Thr Tyr Arg Lys His Pro Ser Thr Pro Leu
    370                 375                 380

Asn Leu Pro Arg Ile Ser Ser Gln Ala Cys Gln Val Asn Gly Tyr Tyr
385                 390                 395                 400

Ile Pro Lys Asn Thr Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg
                405                 410                 415

Asp Pro Asn Val Trp Glu Asn Pro Leu Glu Phe Asn Pro Glu Arg Phe
            420                 425                 430

Met Gly Ala Asn Lys Thr Ile Asp Pro Arg Gly Asn Asp Phe Glu Leu
        435                 440                 445

Ile Pro Phe Gly Ala Gly Arg Arg Ile Cys Ala Gly Thr Arg Met Gly
    450                 455                 460

Ile Val Leu Val Gln Tyr Ile Leu Gly Thr Leu Val His Ser Phe Asp
465                 470                 475                 480

Trp Lys Leu Pro Asn Gly Val Val Glu Leu Asn Met Glu Glu Thr Phe
                485                 490                 495

Gly Leu Ala Leu Gln Lys Lys Ile Pro Leu Ser Ala Leu Ile Thr Pro
            500                 505                 510

Arg Leu Pro Pro Thr Ala Tyr Asn Val Ile Asn Ser Ser
        515                 520                 525
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<211>  1684
<212>  DNA
<213>  gentiana

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gccgccttcc accagggccc accggatggc caatcctagg tgcccttcct cttttgggca     180

acatgccaca tgttactttt gctaacatgg cgaaaaaata tggctcggta atgtacctaa     240
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aaaa 1684

<210> 23
<211> 516
<212> PRT
<213> gentiana

<400> 23

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Met Ser Pro Ile Tyr Thr Thr Leu Thr Leu His Leu Ala Thr Ala Leu
1               5                   10                  15

Phe Leu Phe Phe His Val Gln Lys Leu Val His Tyr Leu His Gly Lys
            20                  25                  30

Ala Thr Gly His Arg Cys Arg Arg Leu Pro Pro Gly Pro Thr Gly Trp
        35                  40                  45

Pro Ile Leu Gly Ala Leu Pro Leu Leu Gly Asn Met Pro His Val Thr
    50                  55                  60

Phe Ala Asn Met Ala Lys Lys Tyr Gly Ser Val Met Tyr Leu Lys Val
65                  70                  75                  80

Gly Ser His Gly Leu Ala Ile Ala Ser Thr Pro Asp Ala Ala Lys Ala
                85                  90                  95

Phe Leu Lys Thr Leu Asp Leu Asn Phe Ser Asn Arg Pro Pro Asn Ala
            100                 105                 110

Gly Ala Thr His Leu Ala Tyr Asn Ala Gln Asp Met Val Phe Ala His
        115                 120                 125

Tyr Gly Pro Lys Trp Lys Leu Leu Arg Lys Leu Ser Asn Leu His Met
    130                 135                 140

Leu Gly Gly Lys Ala Leu Glu Asn Trp Ala Asp Val Arg Lys Thr Glu
145                 150                 155                 160

Leu Gly Tyr Met Leu Lys Ala Met Phe Glu Ser Ser Gln Asn Asn Glu
                165                 170                 175

Pro Val Met Ile Ser Glu Met Leu Thr Tyr Ala Met Ala Asn Met Leu
            180                 185                 190

Ser Gln Val Ile Leu Ser Arg Arg Val Phe Asn Lys Lys Gly Ala Lys
        195                 200                 205

Ser Asn Glu Phe Lys Asp Met Val Val Glu Leu Met Thr Ser Ala Gly
    210                 215                 220
```

Tyr Phe Asn Ile Gly Asp Phe Ile Pro Ser Ile Gly Trp Met Asp Leu
225 230 235 240

Gln Gly Ile Glu Gly Gly Met Lys Arg Leu His Lys Lys Phe Asp Val
245 250 255

Leu Leu Thr Arg Leu Leu Asp Asp His Lys Arg Thr Ser Gln Glu Arg
260 265 270

Lys Gln Lys Pro Asp Phe Leu Asp Phe Val Ile Ala Asn Gly Asp Asn
275 280 285

Ser Asp Gly Glu Arg Leu Asn Thr Asp Asn Ile Lys Ala Leu Leu Leu
290 295 300

Asn Leu Phe Thr Ala Gly Thr Asp Thr Ser Ser Ser Ile Ile Glu Trp
305 310 315 320

Ala Leu Ala Glu Leu Leu Lys Asn Arg Thr Leu Leu Thr Arg Ala Gln
325 330 335

Asp Glu Met Asp Arg Val Ile Gly Arg Asp Arg Arg Leu Leu Glu Ser
340 345 350

Asp Ile Pro Asn Leu Pro Tyr Leu Gln Ala Ile Cys Lys Glu Thr Phe
355 360 365

Arg Lys His Pro Ser Thr Pro Leu Asn Leu Pro Arg Asn Cys Ile Arg
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Gly His Val Asp Val Asn Gly Tyr Tyr Ile Pro Lys Gly Thr Arg Leu
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Lys Ile Asp Pro Arg Gly Asn His Phe Glu Leu Ile Pro Phe Gly Ala
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Asn Arg His Lys Lys Leu Pro Pro Gly Pro Arg Gly Trp Pro Ile Val
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Gly Ala Leu Pro Val Leu Gly Ser Met Pro His Val Thr Leu Ser Arg
    50                  55                  60

Met Ala Lys Lys Tyr Gly Pro Val Met Tyr Leu Lys Met Gly Thr Lys
65                  70                  75                  80

Asn Met Val Val Ala Ser Thr Pro Ala Ala Ala Arg Ala Phe Leu Lys
                85                  90                  95

Thr Leu Asp Gln Asn Phe Ser Asn Arg Pro Pro Asn Ala Gly Ala Thr
            100                 105                 110

His Leu Ala Tyr Asp Ser Gln Asp Met Val Phe Ala His Tyr Gly Ser
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Arg Trp Arg Leu Leu Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly
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Lys Ala Leu Asp Asp Trp Ala His Val Arg Glu Lys Glu Met Arg Tyr
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Val Ala Glu Met Leu Thr Tyr Ala Met Ala Asn Met Ile Gly Gln Val
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Glu Arg Glu Met Lys Ala Leu His Lys Lys Phe Asp Ala Leu Leu Thr
245 250 255

Arg Met Ile Glu Glu His Val Ala Ser Arg Cys His Lys Gly Lys Gly
260 265 270

Asn Tyr Asp Phe Leu Asp Val Val Met Asp His Ser Ser Glu Ser Ser
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Asp Gly Glu Arg Leu Thr Leu Thr Asn Val Lys Ala Leu Leu Leu Asn
290 295 300

Leu Phe Thr Ala Gly Thr Asp Thr Ser Ser Ser Val Ile Glu Trp Ala
305 310 315 320

Leu Ala Glu Met Leu Lys Asn Pro His Ile Thr Lys Arg Ala His Glu
325 330 335

Glu Met Asp Gln Val Ile Gly Lys Asp Arg Arg Leu Lys Glu Ser Asp
340 345 350

Leu Arg Asn Leu Pro Tyr Leu Gln Ala Ile Cys Lys Glu Ala Leu Arg
355 360 365

Lys His Pro Ser Thr Pro Leu Asn Leu Pro Arg Val Ser Ser Gln Pro
370 375 380

Cys Gln Val Asn Gly Tyr Tyr Ile Pro Lys Asn Thr Arg Leu Ser Val
385 390 395 400

Asn Ile Trp Ala Ile Gly Arg Asp Pro Glu Val Trp Glu Asn Pro Cys
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Glu Phe Asn Pro Glu Arg Phe Met Ser Gly Lys Gly Ala Lys Val Asp
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Pro His Gly Asn Asp Phe Glu Leu Ile Pro Phe Gly Ala Gly Arg Arg
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Val Cys Ala Gly Thr Arg Met Gly Ile Val Met Val Gln Tyr Ile Leu
    450                 455                 460

Gly Thr Leu Val His Ser Phe Glu Trp Lys Leu Pro Asn Gly Val Val
465                 470                 475                 480

Glu Leu Asn Met Glu Glu Thr Phe Gly Leu Ala Leu Gln Lys Lys Val
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Cys Val Tyr Gln Ala Asp Tyr Pro Asp Tyr Tyr Phe Arg Ile Thr Lys
        35                  40                  45

Ser Glu His Met Val Asp Leu Lys Glu Lys Phe Lys Arg Met Cys Asp
    50                  55                  60

Lys Ser Met Ile Arg Lys Arg Tyr Met His Leu Thr Glu Glu Tyr Leu
65                  70                  75                  80

Lys Glu Asn Pro Asn Leu Cys Glu Tyr Met Ala Pro Ser Leu Asp Ala
                85                  90                  95

Arg Gln Asp Val Val Val Val Glu Val Pro Lys Leu Gly Lys Glu Ala
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His Leu Ile Phe Cys Thr Thr Ser Gly Val Asp Met Pro Gly Ala Asp
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Tyr Gln Leu Thr Lys Leu Leu Gly Leu Arg Pro Ser Val Lys Arg Phe
145                     150                     155                 160

Met Met Tyr Gln Gln Gly Cys Phe Ala Gly Gly Thr Val Leu Arg Leu
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Ala Lys Asp Leu Ala Glu Asn Asn Lys Asp Ala Arg Val Leu Val Val
            180                     185                     190

Cys Ser Glu Ile Thr Ala Val Thr Phe Arg Gly Pro Asn Asp Thr His
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Leu Asp Ser Leu Val Gly Gln Ala Leu Phe Gly Asp Gly Ala Ala Ala
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Val Ile Val Gly Ser Asp Pro Asp Leu Thr Lys Glu Arg Pro Leu Phe
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Glu Met Ile Ser Ala Ala Gln Thr Ile Leu Pro Asp Ser Glu Gly Ala
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Ile Asp Gly His Leu Arg Glu Val Gly Leu Thr Phe His Leu Leu Lys
            260                     265                     270

Asp Val Pro Gly Leu Ile Ser Lys Asn Ile Glu Lys Ala Leu Thr Gln
        275                     280                     285

Ala Phe Ser Pro Leu Gly Ile Ser Asp Trp Asn Ser Ile Phe Trp Ile
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Ala His Pro Gly Gly Pro Ala Ile Leu Asp Gln Val Glu Leu Lys Leu
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Gly Leu Lys Glu Glu Lys Met Arg Ala Thr Arg His Val Leu Ser Glu
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Arg Lys Lys Ser Ala Glu Glu Gly Ala Ala Thr Thr Gly Glu Gly Leu
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Lys Val Gly Ala Trp Gly Met Ala Val Ala Ser Thr Pro Ala Ser Ala
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2003/001111

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C12N 15/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See "electronic data base" box below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See "electronic data base" box below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, WPIDS: flavonoid 3',5' hydroxylase; DGENE (blast) : SEQ ID NO: 5, 10, 12, 14, 16, 18, 21, 27, 30, 32

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 632 128 A1 (KYOWA HAKKO KOGYO CO., LTD.) 4 January 1995 See Claim 1; pages 43-46 [shares 68% identity with SEQ ID NO: 12; 66% with SEQ ID NO: 14; 65% with SEQ ID NO 16; 58% with SEQ ID NO: 21]	1-86, 89-92
X	EP 0 522 880 B1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 21 March 2001 See Fig. 10 [shares 69% identity with SEQ ID NO: 12; 66% with SEQ ID NO: 14; 58% with SEQ ID NO: 21, 73% with SEQ ID NO: 27, 64% with SEQ ID NO: 32]; Fig 9 [shares 65% with SEQ ID NO: 16]	1-86, 89-92
X	WO 2000009720 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 24 February 2000 Specification	1-6, 11, 29-32, 35, 40, 45, 49, 57-82, 89-92

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 January 2004	22 JAN 2004

Name and mailing address of the ISA/AU	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2003/001111

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1996036716 A1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 21 November 1996 Specification	1-6, 11, 29-32, 35, 40, 45, 49, 57-82, 89-92
X	WO 1993020206 A1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 14 October 1993 See Fig 3A-D [shares 68% identity with SEQ ID NO: 12; 67% with SEQ ID NO: 14; 65% with SEQ ID NO: 16; 58% with SEQ ID NO: 21; 72% with SEQ ID NO: 27; 63% with SEQ ID NO: 32]	1-86, 89-92

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2003/001111

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	0632128	AU	29560/92	CA	2130800	CA	2365599
		US	6114601	US	6232109	US	2002100072
		WO	9318155				
EP	0522880	AU	19530/92	AU	22733/92	AU	67895/94
		CA	2112373	CA	2163220	CN	1071456
		CN	1127015	EP	0703982	IE	922272
		JP	2000023686	NZ	243500	NZ	266401
		PL	298239	PL	311691	SG	45175
		SG	45187	US	5349125	US	5569832
		US	5861487	US	5948955	WO	9301290
		WO	9428140	ZA	9205180		
WO	0009720	AU	53815/99				
WO	9636716	AU	56396/96	CA	2202668	EP	0873410
		HU	9802555	NZ	307119	US	6080920
WO	9320206	AU	37413/93	CA	2132961	EP	0640136
		NZ	249808	US	5639870		

END OF ANNEX